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Role of testosterone in prevention of Alzheimer's Disease

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Role of testosterone in prevention of Alzheimer's Disease

This thesis is presented for the degree of

Doctor of Philosophy

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Chapter 1

The role of testosterone and luteinizing hormone (LH) in subjective memory complainers (SMC)

1.1 Background

Alzheimer's disease (AD), is the most common cause of dementia in the elderly and is characterized by a progressive memory decline, impairments in language and visual-spatial skills, impairments in behaviour, results in a loss of independence, reduction of quality of life, and ultimately death. Although our knowledge and understanding of the disease continues to grow, currently there are no effective treatments for reversing or even stabilizing the process of the disease. AD remains perhaps the most devastating disease of older people and constitutes a large social and economic burden to both the families and society as a whole. Currently there are 30 million individuals worldwide with dementia (Ferry, C.P *et al.*, 2005) and some predictions indicate as much as a 4 fold increase in the prevalence of AD by 2050 (Brookmeyer *et al.*, 2007). Thus, there is an urgent need for better tools for diagnosis, prevention, and treatment to avoid this upcoming epidemic.

Age is the most important known risk factor of AD. As people age, there is a normal decline in the physiological functions of their body, in particular the hormonal deficiency following reproductive senescence known as menopause in women and andropause in men. A number of studies involving postmenopausal women have shown a correlation between decreasing levels of estrogen and increased levels of beta amyloid ($A\beta$), the major component of the neuritic plaques found in the AD brain. Although the role of testosterone in men has received less attention, it has been shown to share a similar action to that of estrogens on the generation of the $A\beta$. Therefore, it has been suggested that testosterone may have a similar role to estrogen in the relation to AD.

At present, a number of studies in the AD field focus on testosterone levels in men and its effects on the nervous system. While the results are preliminary and the role of androgens in the aged-related cognition is still poorly understood compared to those studies of estrogen, it has been proposed that testosterone therapy may delay the onset of AD in elderly men.

Luteinizing hormone (LH), one of the pituitary gonadotropin hormones, is synthesized and secreted under the regulation of gonadotropin-releasing hormone (GnRH), pituitary factors and gonadal hormone feedback which collectively are termed the Hypothalamus-Pituitary-Gonadal (HPG) axis. LH is secreted in a pulsatile manner from

pituitary into the peripheral circulation which eventually affect target organs expressing the LH receptor (LHR). In the gonads, LH stimulate gonadal hormone production, such as testosterone, estrogen, progesterone, activin, and inhibin. LHR is highly expressed in the hippocampus; a brain region involved in memory function and which is severely affected in AD (Barron, A.M *et al.*, 2006). Several studies also confirmed that the brain may also be a target organ for LH (Lei, Z *et al.*, 1993; Al-Hader, A.A *et al.*, 1997). However, the regulation of LHR in the brain has been largely unexplored.

In aging men, changes of LH levels have been reported with the levels of serum LH shown to be two to three times higher than men in their mid-20s (Neaves, W.B *et al.*, 1984). Recent studies by Short *et al.*, 2001 showed that high levels of LH, are associated with AD. However, whether LH plays a direct role in AD pathogenesis still remains unclear. Recent studies have linked testosterone depletion and perhaps LH elevation with increased risk for the development of AD in men. It is unclear whether the altered actions of both testosterone or LH contribute to the pathogenesis of AD. It is unlikely that any one of the hormones from HPG axis plays a single and predominant role in the reproductive system but rather it may be a combination of hormones acting in concert.

This chapter will review the current understanding of the characteristics and pathogenic mechanisms of AD, focussing on the relationship between testosterone, LH and AD. In addition, the review will also discuss the benefits of testosterone and LH suppression in AD.

1.2 Alzheimer's Disease : Epidemiology and Clinical Characteristics

Alzheimer's disease (AD) is the most prevalent form of dementia, accounts for about 50-60% of all dementia cases, compared to other types of dementia, such as vascular dementia (15-20% of the cases), Lewy body dementia (up to 20% of the cases) and fronto-temporal dementia (approximately about 10% which is associated with a younger age of onset) (Schulz, R., Noelker, S Linda., Rockwood, K., Sprott, R, 2006). The number of cases with dementia as well as AD world-wide will increase as the population grows, affecting approximately 4-5 million people in the United States and around 15 million people worldwide (Hebert *et al.*, 2003). Other developed countries

such as Australia and Canada will undergo a similar pattern. Although AD is known to be a major problem in developed countries, it already poses a great problem in developing countries, predominantly in Asian countries where the impact of this disease is predicted to be catastrophic if effective prevention measures are not implemented in the near future (Ferri *et al.*, 2005). Moreover, in Indonesia, as a developing country with a high rate of population growth, AD cases are projected to have 295% increment between 1990 and 2030 (Draper and Brian, 2004). The decline in fertility and improvements in life expectancy have contributed to the aging population in Indonesia. As seen in western countries there is a predominance women compared to men in the older age groups which may contribute further to the dementia epidemic. Furthermore there is a greater degree of aging in rural areas and taken together with the large rural population in Indonesia and its associated lower socio-economic status the prevalence of this disease will be considerable. The absolute number of people in Indonesia has increased from 4.9 million in 1950 to 16.3 million in 2000. By 2050, it is estimated that one in four Indonesian would be classified as an elderly person compared to one in ten at present, resulting in 73.6 million in this population group (Fletcher Robert., 2010). Currently there are very few facilities for the treatment and care of people with Alzheimer's disease and the government is poorly aware of the magnitude of this problem and is thus unprepared to deal with the looming dementia epidemic and no initiatives have yet been taken to develop early diagnostic and prevention programs to combat the consequence of this devastating disease.

AD was first described in 1907 by Alois Alzheimer, a German physician who described the neuropathology in the brain of a 51 year old woman named Augusta D. After her death, an autopsy revealed dense deposits outside and around nerve cells in her brain, and twisted strands of fibre inside dead neurons, which were later known as neurofibrillary tangles and neuritic plaques, respectively. AD is characterized clinically by the insidious onset and progressive decline of mental functions affecting long-term episodic memory (Henderson, 1997). However, AD cannot be definitively diagnosed until post-mortem examination of the brain for the neuropathological hallmarks of AD. (Mirra *et al.*, 1991). It was later discovered that the major component of neuritic plaques is beta amyloid ($A\beta$), a peptide proteolytically derived from the larger amyloid precursor protein (APP) (for review see Nunan and Small, 2000, Glenner and Wong,

1984; Masters *et al.*, 1985). Although more than a century has passed since Alois Alzheimer's findings and research has been carried out for decades around the world, there is still many questions directed at understanding the pathogenesis of AD and there is no effective treatment.

AD attacks several different regions of the brain including the cerebral cortex, which is involved in conscious thought and language; the basal forebrain, which is important in memory and learning; and the hippocampus, which is fundamental to memory storage. AD can be divided into two forms based on the age of onset, i.e. early onset (EOAD; the age of onset before 65 years old) and late onset (LOAD; the age of onset after 65 years old). EOAD is the more severe form but is less common and accounts for ~5-10% of the cases. In some cases, EOAD is caused by autosomal dominant, inherited mutations identified in three different genes, the amyloid precursor protein (APP) gene (Goate *et al.*, 1991; Murrell *et al.*, 1991), the presenilin-1 (PS1) gene (Sherrington *et al.*, 1995) and presenilin-2 (PS2) gene (Levy-lahad *et al.*, 1995; Rogaev *et al.*, 1995); and characterized by the increased production of A β , in particular the longer form and more toxic A β ₄₂ (for review see Kowalska, 2003).

On the other hand, LOAD is the most common form of the disease, which accounts ~95% of the cases, and is characterized by several risk factors including age, the presence of Apolipoprotein E ϵ 4 allele (APOE ϵ 4) (Strittmatter *et al.*, 1993; Martins *et al.*, 1995; Scheuner *et al.*, 1996), brain trauma, diet (i.e. high levels of cholesterol), low levels of testosterone and estrogen (Corder *et al.*, 1993; Haskell, Richardson and Horwitz, 1997; Short *et al.*, 2001) and more recently increased levels of gonadotropins i.e. luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Bowen *et al.*, 2002; Short *et al.*, 2001).

Each of the major neuropathological hallmarks of AD, namely the senile plaques, neurofibrillary tangles, neuronal atrophy or neuronal death, and cerebral amyloid angiopathy, (Dickson, 1997; Braak, H., and Braak, E., 1991; Poirier, 2005) will be described below.

Senile plaques

Senile plaques (SP) or amyloid plaques are composed of beta amyloid protein ($A\beta$) in the central core surrounded by reactive astrocytes and microglia found in the brain of Alzheimer's disease and normal aging (Figure 1.1). As people get older, from the age of 60 years, the proportion of the people with plaques increase linearly. In AD, amyloid plaques develop in vulnerable regions of the brain, such as hippocampus, which plays an important role in the formation of short and long-term memory. Amyloid plaques have variable shape and size but are on the average 50 μ m (Franke, M., 1976). In addition, Amyloid plaques also spreads over time in a variety of directions into the cerebral cortex, the area that is involved in processing of sensory input and learning (Rodgers, 2003).

According to the amyloid hypothesis (Selkoe,D.J & Hardy,J., 2007) which was proposed over 25 years ago, accumulation of beta amyloid in the brain is the primary cause of AD pathogenesis. But recently, it is currently unclear whether amyloid plaques are actually the primary cause of Alzheimer's disease (AD) or the result of an another initiating factor. Evidence in support of this notion includes the observation that dense plaques accumulate with age, even in people who have no cognitive impairment. Furthermore, plaques do not correlate with disease severity (Robert,D *et al.*,1991). In addition, $A\beta$ is also deposited in the exterior of neurons in several unrelated disorders.

On the other hand, the strongest evidence in support of $A\beta$ playing a key role in the onset of AD may be found in the genetic studies of families with autosomal mutations that cause AD and are all shown to result in increased production of $A\beta$. Interestingly it has been suggested that smaller aggregates of $A\beta$ (ie oligomers) and not the amyloid plaques *per se* play the major role in the damaging effect on dendrites (Moolman *et al.* 2004;Gandy, S *et al.*, 2010; Selkoe, D.J, 2000). Furthermore, while amyloid plaques *per se* do not correlate with the severity of the disease there is strong evidence to demonstrate that soluble $A\beta$ levels, which lead to the development of the amyloid plaques do correlate with disease severity. Additional genetic evidence for the role of amyloid in AD is found in patients with Down syndrome (trisomy 21) and it is proven that most people with Down syndrome will go on to develop AD (Wisniewski *et al.*, 1985).

Neurofibrillary tangles

Neurofibrillary tangles (NFT) are intracellular lesions composed of abnormal insoluble filaments which is paired helical filaments and straight filaments of abnormally phosphorylated tau protein accumulates within the neuron (Goedert *et al.*, 2002). Tau is a microtubule associated protein found in neuronal axons throughout the central and peripheral nervous systems and to a lesser extent in glial cells. Tau protein is a potent promoter of tubulin polymerisation and microtubule stabilisation, thus serving an important role of neuronal outgrowth and morphology (Lovestone and McLoughlin, 2002). There are six isoforms of tau in CNS, differing by possession of N-terminal inserts of unknown function and by possession of three or four imperfect repeats that bind to microtubules (Goedert *et al.*, 1996). NFTs are found predominantly in neuronal cell bodies surrounding SPs. But NFTs also may occur without SPs (Shukla and Bridges, 1999). The number and distribution of NFTs shows a moderate correlation with the severity of AD (Schonheit *et al.*, 2004).

The hyperphosphorylation of tau has not only been associated with AD but also with a number of other neurological diseases such as fronto-temporal dementia, Pick's disease, cortico-based degeneration, and progressive supranuclear palsy (Goedert *et al.*, 2006). However, the mechanism that trigger the tau hyperphosphorylation are still unclear.

As mutations in tau give rise to tangles but mutations in APP give rise to both plaques and tangles, it follows that A β must, biochemically, precede tau pathology in AD. This is consistent with the studies on Down's syndrome brain where it was shown that amyloid deposition preceded tangle aggregation REFS. The relative importance of A β deposition and neurofibrillary tangles in the onset of dementia in AD has been a subject of intense debate between amyloid and tau proponents, known as "baptists" and "tauists" respectively (Hardy and Selkoe, 2002). However, recently there are some *in vitro* and *in vivo* findings which suggested that both A β and tau play important roles in AD (LaFerla, F.M, 2008; Wilson, D.M and Binder, L.I, 1997; Eckert, A *et al.*, 2010).

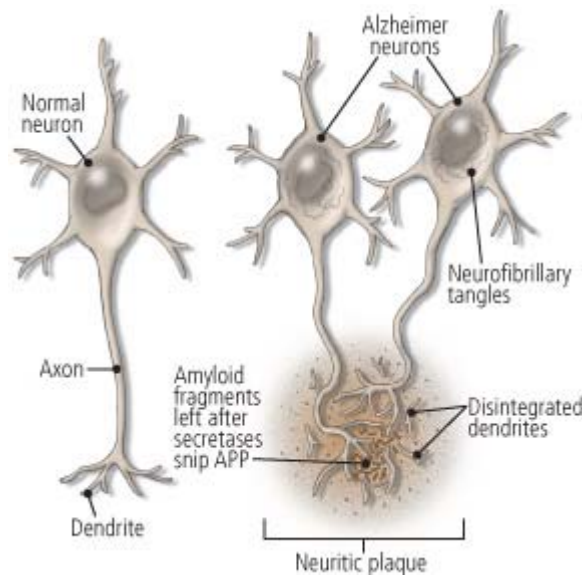


Figure 1.1 : Senile plaque and neurofibrillary tangle. Image courtesy of www.maryjosephfoundation.blogspot.com

Cerebral amyloid angiopathy (CAA)

Cerebral amyloid angiopathy (CAA) is an accumulation of amyloid in cerebral and meningeal blood vessels. CAA is observed in more than 80% of AD brains (Attems *et al.*, 2007). However, some level of CAA can also be found in the brains of non-demented elderly. There is now considerable evidence that CAA is a major factor in the pathogenesis of AD (Fryer *et al.*, 2005; Martins *et al.*, 2006 (Maia *et al.*, 2007)

1.2.1 A β and Alzheimer's disease

A β is the major protein component of neuritic plaques in the brain of AD patients. A β , a 4kDa small oligopeptide, is generated from the proteolytic processing of its much larger, 100-130 kDa parent molecule, the amyloid precursor protein (APP) (Glenner and Wong, 1984, Masters *et al.*, 1985), the gene for which is located on chromosomes 21, expressed in brain and in several peripheral tissues such as testis, ovary, liver, kidney, spleen, pancreas, and salivary gland (Johnson-Wood *et al.*, 1997); Beer, J *et al.*, 1995;

Bates *et al.*, 2009). A β which varies in length from 39 to 42 residues is not the result of abnormal or pathological APP processing, as was originally believed, but it is secreted constitutively by normal cells in culture (Haas *et al.*, 1992; Shoji *et al.*, 1992) and can be detected in plasma and CSF of healthy humans (Seubert *et al.*, 1992).

A β generated in intracellular organelles can be secreted into the extracellular space. The most common isoforms are A β_{40} and A β_{42} ; the shorter form is typically produced by cleavage that occurs in the endoplasmic reticulum, while the longer form is produced by cleavage in the trans-Golgi network. The A β_{40} form is the more common of the two, but A β_{42} is the more fibrillogenic and is thus associated with disease states. The major species of secreted A β is the soluble A β_{40} found in the CSF at low nano-molar concentrations (Vigo-Pelfrey, *et al.*, 1993). Although A β is soluble in biological fluids and tissue at physiological concentrations (Mulnard *et al.*, 2000 ; Shumaker *et al.*, 2003), at higher concentrations it aggregates to form extracellular deposits found in AD plaque (Lenner and Wong, 1988) . A β_{42} is dominantly found in amyloid plaques of AD brains and is thought to seed the deposition of A β_{40} . These amyloidogenic proteins have characteristics such as being insoluble in water and having a high β -sheet secondary structure that is associated with a tendency to aggregate or polymerize. Ultrastructurally the deposits are mainly fibrillar and show characteristic apple-green birefringence when viewed under polarized light after Congo red staining (Asami-Odaka *et al.*, 1995; Wisniewski, and Frangione, 1992; Goodenough, Engert and Behl, 2000).

A β is secreted from CNS into the extracellular space via transport through the blood-brain barrier (BBB) and/or via the ISF bulk flow, allowing its detection in the CSF and plasma (for review see Zlokovic, 2004) and its oligomers are stable molecules that can exist for long periods in the brain prior to conversion to fibrillar structures (Chromy, *et al.*, 2003). It is of particular interest to understand which form of A β is toxic to neurons. A study by Dodart *et al.*, 1999 showed that it is not the number or the concentration of A β plaques that predict the extent of memory deficits but the levels of soluble A β peptides. In addition, soluble A β peptide are also implicated in spatial learning deficits in APP transgenic mice (Koistinaho *et al.*, 2001).

The fundamental mechanisms underlying the neuronal toxicity of A β are complex and not well understood yet. A β toxicity may be induced by two proposed pathways : the first pathway implies that A β directly causes injury to the cell by membrane damage (Mark, *et al.*, 1997) while in the second pathway A β acts indirectly by enhancing neuronal vulnerability to neurotoxic insults, such as excitotoxicity, hypoglycaemia, oxidative stress or metabolic impairment (Arias *et al.*, 2002; Matson *et al.*, 1992). These different pathways may result from the action of different forms of A β . This is consistent with not only the aggregated forms of A β being neurotoxic (Lorenzo, *et al.*, 1994), but also the overwhelming evidence in support of soluble oligomeric forms being neurotoxic (Roher, *et al.*, 1996; Etcheberrigaray, E *et al.*, 1994, Walsh *et al.*, 2002; Wang *et al.*, 2002).

A β not only possesses neurotoxic properties but is also shown under specified conditions to exhibit neurotrophic properties (Rismann *et al.*, 2002). A β is not toxic to undifferentiated neurons regulated by cyclin dependent kinase 5 (Cdk-5) but toxic to differentiated neurons (Koo, *et al.*, 1993; Koo *et al.*, 1994). In addition, recent findings by Verdile *et al* (personal communication) indicates that A β production increases only in the late event of the neuronal cell cycle.

1.2.2 The Amyloid Precursor Protein (APP)

APP is an integral membrane protein, with a small intracellular domain and a large extracellular domain; it is hypothesized that the extracellular domain mediates cell-cell signalling, while the intracellular domain relays those signals to the rest of the cell. APP has an important role in helping neurons grow and survive and also may help damaged neurons repair themselves, especially after brain injury (Rodgers, 2003). It also plays a role in cell adhesion, apoptosis, cell signalling, and as a cell surface receptor (for review see Zheng and Koo, 2006). APP is widely expressed in cells throughout the body where the amount produced is influenced by the developmental and physiological state of the cells. There are 10 isoforms of APP ranging from 563 to 770 amino acids. Of these

isoforms APP 695 is the most abundant form in the brain, produced mainly by neurons (Matson, M.P., 1997).

The generation of A β from APP is complex, as the APP protein is cleaved several times in the cell. APP is normally cleaved by three secretase genes, called α , β , or γ -secretase. The proteolytic cleavage of APP, a type I transmembrane protein, is metabolized by two competing pathways which are the amyloidogenic and the non-amyloidogenic pathways (Figure 1.2). In the non-amyloidogenic pathway, APP is processed by an α -secretase that cleaves within the A β region (thus precluding its formation), resulting in the release of a soluble ~110–120 kDa N-terminal APP fragment (sAPP α) from the cell surface. This pathway also releases a C-terminal fragment (CTF) that is 83 amino acids in length (C83) which remains in the cell membrane with a relative long half life and can be detected to different extents in metabolically labelled cells (Oltersdorf *et al.*, 1990). The C83 fragment can also be cleaved by a γ -secretase to release a small, non-toxic 3 kDa fragment known as p3. (Siman, R., Mistretta, S., Durkin, J.T., *et al.*, 1993). Cleavage of APP by α -secretase is the major proteolytic pathway, and there are candidates for α -secretase such as ADAM10, ADAM17, ADAM 19, and TACE (Sangram, S.S *et al.*, 2007). Although recent studies suggest that the constitutively active α - secretase is ADAM 10 (Lichtenthaler, 2011). Only a small fraction of the total APP is cleaved by α -secretase in most cell types, resulting in the intact of APP (Strooper and Annaert, 2000). Cholesterol is thought to modulate the APP processing pathways. When cholesterol levels are low APP is preferentially metabolized via the non-amyloidogenic pathway where it is cleaved by α -secretase whilst at high cholesterol levels APP is metabolized by β -secretase (BACE) and γ -secretase through the amyloidogenic pathway (Kojro, E *et al.*, 2001).; Ian, J.M *et al.*, 2009).

In the amyloidogenic pathway, APP is cleaved first at the N-terminus of the A β peptide sequence by β -secretase (BACE) releasing an APP β fragment and leaving a 99 amino acid CTF embedded in the membrane (C99). The C99 fragment is then subsequently cleaved by γ -secretase, within its intra-membrane region to release the A β peptide and AICD. The result is the generation of A β ₄₀ and A β ₄₂. BACE has been identified as the protein that contains β -secretase activity and homologues to the pepsin family of

aspartyl proteases (Vassar, R *et al.*, 1999). The BACE-1 protein appears to be the major enzyme in the amyloidogenic pathway and is highly expressed in neurons (Fluhrer, R *et al.*, 2002).

γ -secretase is a protein complex within the lipid bilayer that cleaves within one of the transmembran regions of the APP, which is the C-terminal end of the A β sequence and it is formed by presenilins (PS), nicastrin, Aph-1 and Pen-2 (Francis, R *et al.*, 2002). The participation of PS in the pathogenesis of AD can be seen in many cases of familial AD (FAD) (see review of Davis, J.N *et al.*, 1997). The cellular sites of γ -secretase activity remain controversial. Study by Kaether *et al.*, 2006 found that intracellular generated by γ -secretase is at the plasma membrane and/or early endosomes (Kaether *et al.*, 2006).

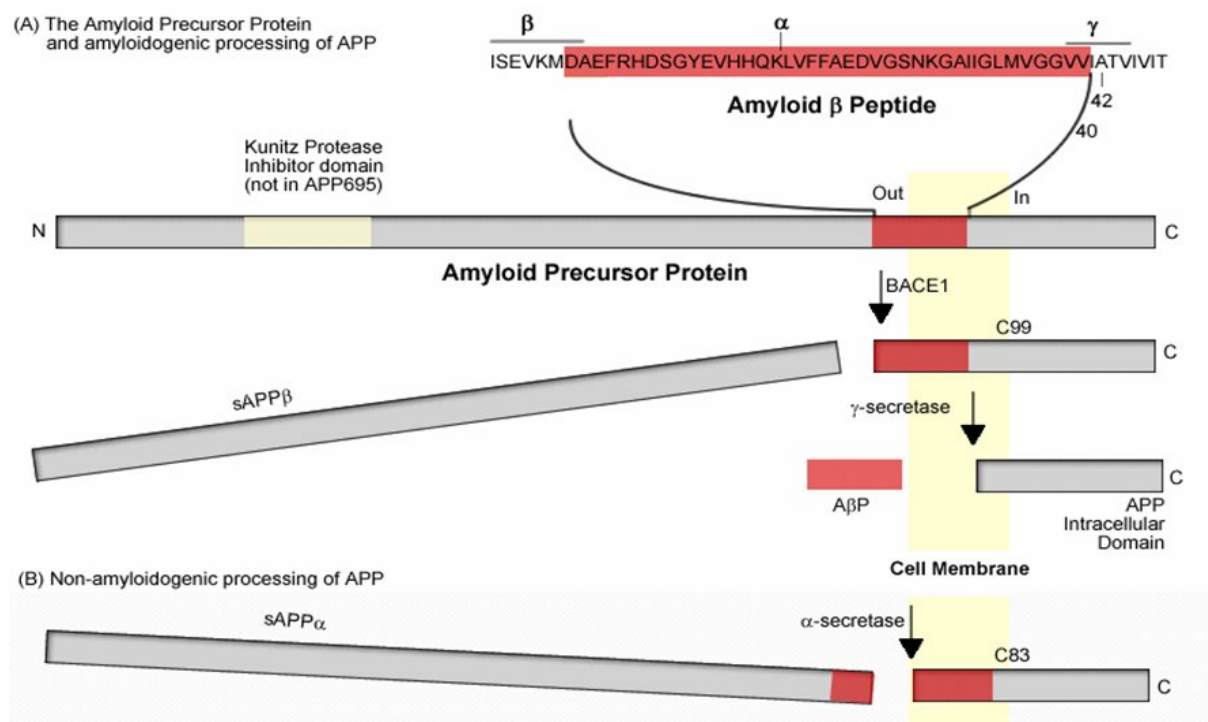


Figure 1.2 : Schematic representation of APP processing. The proteolytic cleavage of APP resulted in two pathways : the amyloidogenic and non-amyloidogenic pathways (kngreen.googlepages.com)

Changes in the activity of the secretase proteins can impact on A β genesis. If α -secretase activity decreases, or β -secretase activity increases, more A β will be formed. As expected, several mutations in these secretases have been found in human AD patients that affect secretase activity. In addition, mutations in APP itself can favor the generation of the A β_{42} product, which is more pathogenic than the A β_{40} product. Interestingly, α and γ cleavage appears to occur extracellularly, while BACE and γ cleavage occurs entirely in the endosomal-lysosomal compartment (Kaether *et al.*, 2006). Some studies also show that the mis-sense mutations in PS1 and PS2 genes, which can often be found in families with early onset AD, result in the increased production of A β_{42} peptide as opposed to the less amyloidogenic A β_{40} form of the peptide (Selkoe, 1998). In the Alzheimer's Disease & Frontotemporal Dementia Mutation Database by July there are 25 pathogenic mutations in APP, 155 in PS1, and 10 in PS2 (Cruts and Rademakers, 2006; <http://www.molgen.ua.ac.be/ADMutations/>).

1.3 ApoE and Alzheimer's disease

Human apolipoprotein E (apoE [protein], APOE [gene]) is located on the long arm of chromosome 19q 13.2 (Olaisen, Teisberg and Gedde-Dahl, 1982; Das *et al.*, 1985; Strittmatter *et al.*, 1993) with a molecular mass 34.2 kDa and is synthesized mainly in the liver, and the brain, as well as circulating cells including macrophages and monocytes (Siest, G, *et al.*, 1995). ApoE plays an important role in the periphery as a mediator of lipoprotein metabolism and lipid clearance. In the central nervous system its importance is underscored by the low abundance of other apolipoproteins and plays a role in the redistribution of lipid and cholesterol during membrane repair and believed to be important for maintaining synaptic plasticity especially after neuronal injury. One of the first indication that apoE might be involved in AD came from the immunochemical localization of apoE in extracellular amyloid plaques and in neurons containing neurofibrillary tangles (Wisniewski, T *et al.*, 1992).

ApoE serves as a ligand for low density lipoprotein receptor (LDLR and LDLRP). The three major isoforms of APOE : E2, E3, and E4, are encoded by distinct alleles ϵ 2, ϵ 3, and ϵ 4 and therefore have six phenotypes (Utermann *et al.*, 1980); Zannis *et al.*, 1982. They differ in having cysteine (Cys) or arginine (Arg) at amino acid positions 112 and

158 and vary in their metabolic properties (Mahley, 1988). ApoE2 (Cys¹¹², Cys¹⁵⁸) binds defectively to low density lipoprotein (LDL) receptors. ApoE3 (Cys¹¹², Arg¹⁵⁸) binds normally to LDL receptors and is associated with normal lipid metabolism. ApoE4 (Arg¹¹², Arg¹⁵⁸) has a relative stronger affinity than apoE3 for LDL receptors and is associated with elevated cholesterol levels (for review see Raber, 2004). This isoform was first shown to be a risk factor and an increased risk for AD since 1993 (Strittmatter *et al.*, 1993), enhanced A β aggregation, increased A β deposition, and reduces A β clearance (Schemechel, D.E *et al.*, 1993; Berr, C *et al.*, 1994), whereas APOE2 has a protective effect (Corder *et al.*, 1993) compared to APOE3 (the most common isoform).

Since it was reported that the apoE ϵ 4 allele is overpresented in AD patients, (Strittmatter *et al.*, 1993) several studies have examined the relation of apoE alleles to AD pathology. Studies have shown that apoE associates with high avidity to soluble A β (Wisniewski *et al.*, 1993; Raber *et al.*, 2004). ApoE is also known to co-localise with A β plaques (Holtzman, 2002), and APOE ϵ 4 is associated with increased plaque density and size (Schmechel *et al.*, 1993; Holtzman, 2002). In clinical studies of AD, testosterone levels in subjects without the APOE ϵ 4 allele were lower in AD cases than in controls (Hogervorst *et al.*, 2002). This study also found that cognitively normal APOE ϵ 4 carriers had lower testosterone levels than individuals without the allele (Hogervorst *et al.*, 2002), suggesting that APOE status can affect testosterone levels.

The mechanism whereby apoE4 promotes AD is not yet established, perhaps by accelerating plaque formation or by its association with poor neuronal repair. Considerable evidence supports both mechanisms. *In vitro* study showed all apoE isoforms inhibit A β aggregation with apoE4 less effectively than apoE3 (Ma *et al.*, 1994, Moir *et al.*, 1999, Sanan *et al.*, 1994), suggesting a way in which ϵ 4 may increase amyloid deposition. In addition, transgenic amyloid-producing mice expressing apoE4 develop less A β deposition than apoE knockout mice (Bales *et al.*, 1997). These observations consistent with an effect of apoE isoforms on A β aggregation in AD. AD patients with at least one ϵ 4 allele tend to have more amyloid deposition than do subjects without APOE ϵ 4 (Beffert and Poirier, 1996; Beffert *et al.*, 1999).

With regard to AD development, the effect of APOE ϵ 4 while dose-dependent, in terms of increased risk is not definitely deterministic of developing AD. (Corder, E.H *et al.*, 1993). At present, the exact mechanism by which APOE ϵ 4 affects the pathophysiology of AD is not clear.

Plasma apoE is primarily derived from liver parenchymal cells and from macrophages throughout the body (in a much lesser extent) whilst apoE in the CNS is known to be derived exclusively from the brain (Linton, M.F *et al.*, 1991). According to several studies, A β is directly bound by apoE as demonstrated *in vitro* (Whittemore, E. R., Loo, D. T. and Cotman, C. R., 1994; Strittmatter, W. J., Weisgraber, K. H., Huang, D. Y., *et al.*, 1993; Wisniewski, T, Frangione, B, 1993) and has been shown to co-localize with A β in AD plaques (Wisniewski, T. and Frangione, B, 1992; Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E. and Ikeda, K, 1991). There are conflicting reports over the effects of apoE isoform specific differences on interactions with A β . Strittmatter *et al.*, 1993 reported that apoE4 binds A β ₄₀ more rapidly than apoE3. In contrast, LaDu *et al.*, 1995 reported the opposite binding preference of apoE in HeK-293 cells, with an apoE3-A β ₄₀ complex occurring more readily than the apoE4-A β ₄₀ complex over a wide pH and concentration range. Subsequent studies by several groups including our own have validated and extended the finds of LaDu *et al* demonstrating that apoE2 and apoE3 binds avidly to A β while apoE4 binds poorly if at all under native conditions where apoE is fully lipidated unlike the original experiments of Strittmatter *et al.*, 1993 Though many studies have examined the relation of ϵ 4 to AD pathology, the exact role of apoE is yet to be elucidated.

It is not yet known whether different isoforms of apoE have different clearance activities. One of the expectations is that apoE4 may have a significantly reduced clearance activity when compared to others apoE isoforms. Yang *et al.*, 1993 first demonstrated that under *in vitro* conditions apoE cleared A β in an isoform specific manner such apoE2 was most efficient in clearing A β followed by apoE3 with apoE4 showing virtually no clearance. Subsequently Sharman *et al.*, 2010 shows that APOE genotype significantly altered the peripheral clearance of A β ₄₂ in transgenic mice , indicating that apoE isoforms may impact on clearance of A β from the brain.

1.3.1 Cholesterol and Alzheimer's Disease

The brain must synthesize its own cholesterol *de novo*, since cholesterol cannot pass the BBB (Papadopoulos, 1993; Bjorkhem *et al.*, 2006). Therefore, regulation of cholesterol synthesis and metabolism in the brain is largely independent of changes in the periphery (Bjorkhem *et al.*, 2006). Nevertheless, there is increasing evidence from clinical, epidemiological, and laboratory studies showing that changes in cholesterol metabolism play a role in the pathogenesis of AD and that brain cholesterol as well as plasma cholesterol metabolites may act as clinically relevant biomarkers (Bjorkhem *et al.*, 2006).

Cholesterol metabolism also appears to play an important role in the biology of APP and A β production (Simons *et al.*, 1998). Interestingly, elevated plasma cholesterol levels were reported in individuals carrying APOE4 (Sing and Davignon, 1985; Ehnholm *et al.*, 1986), which is the major genetic risk factor for AD (Corder *et al.*, 1993; Poirier *et al.*, 1993). However, Wolozin *et al.*, 2006 concluded that the link between AD and APOE4 was not directly related to cholesterol but the overall literature indicates a close relationship between apoE4, cholesterol metabolism and the risk of AD. In conclusion, AD may represent an interaction between several different factors that together, contribute to its ultimate phenotype. More recently sex hormones such as testosterone have been shown to alter A β production and mediate alterations in cardiovascular risk factors particularly cholesterol metabolism. The following section outlines age-related changes in testosterone and gonadotropins and describes mechanisms by which these hormones contribute to AD pathogenesis.

1.4 Testosterone and its role in Alzheimer's disease risk and pathogenesis.

1.4.1 Testosterone

Testosterone is the predominant androgen in males. Though its a hormone originally associated with the modulation of reproductive function and secondary sexual characteristics, testosterone is now recognized to also have a potent function in the structure and function of the brain as well as behaviour and cognitive function. More than 95% is secreted by the testis, which produces approximately 6-7 mg per day, about 16

2500 mg each year. The normal range of plasma testosterone in males is debateable (Carruthers *et al*, 2007), and varies between populations, but is generally taken to be 260 to 1080 ng/dl² or 8.8-36.7 nmol/L which declines by age 80 to 50% of the average value of age 20. In females, the circulating testosterone levels are typically about 10% of those observed in men (Coffey, 1988).

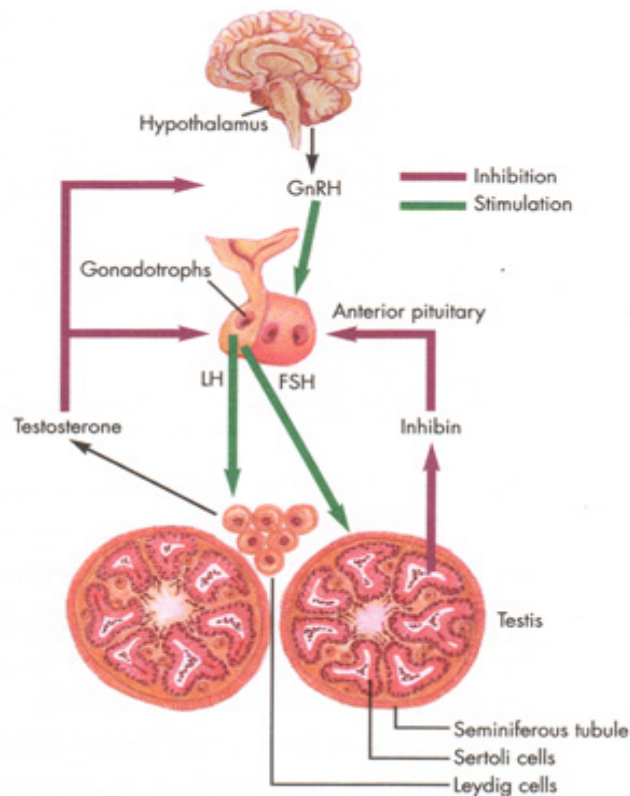


Figure 1.3 : Male Hypothalamus-Pituitary-Gonadal (HPG) Axis

(<http://www.homefertility.com/hypothalamus.jpg>)

In the normal male, testosterone is produced as follows (Figure 1.3). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus. This hormone acts on the pituitary to secrete Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH). LH then stimulates the testicular Leydig cells to produce testosterone in a pulsatile manner with a peak in the morning and lowest level in the evening. Testosterone itself negatively inhibits GnRH release from the hypothalamus, whilst

FSH stimulates the testicular Sertoli cells and seminiferous tubules to promote spermatogenesis. A Sertoli cell produced hormone to reduce FSH secretion known as inhibin. Testosterone production increases rapidly at the onset of puberty, but starts to drop as a man approaches 30 years of age. This is further discussed in section 1.4.3

In young males, testosterone acts on a number of other organ systems besides the testes, including muscle, bone, central nervous system, prostate, bone marrow, (see review of Tenover J.L, 1994). Testosterone within the bloodstream occurs in two forms : free and bound. Roughly 2% of total testosterone is made up of free testosterone, which is the most bioavailable form, and can be transported across the BBB into the CNS . The majority is bound to sex hormone-binding globulin or SHBG (44%) and albumin (44%) (Bates *et al.*, 2005). Free testosterone diffuses passively through the cell membranes into the target cell, where it binds to the specific androgen receptor (AR). The serum free testosterone and the testosterone that binds to albumin are readily available for biological action. As a matter of fact, albumin-bound testosterone dissociates during tissue transit, whereas the strong binding of testosterone to SHBG will usually not allow for substantial dissociation during the tissue transit time (Tenover J.L, 1994). The combined free and albumin-bound testosterone (the non-SHBG bound testosterone) is often referred to as the “bioavailable testosterone”. However, the testosterone that is actually available for biological action may vary according to the tissue and pathophysiological condition, and at present a reliable androgen marker is still lacking.

Although testosterone binds to the nuclear AR in target cells, its activity is significantly less than the more active androgen, dihydrotestosterone (DHT), and is not included in the fraction of testosterone which can be aromatized into estradiol in tissues expressing P450 aromatase enzyme. Hence, testosterone activity is determined by testosterone concentrations, α -reductase and aromatase activity in the amount of available tissue such as adipose tissue that converts it to estrogen, together with the level of expressions of the androgen receptor (AR) (Janowsky, J.S, 2006).

Male aging is associated with a gradual, progressive decline in serum levels of total testosterone, bioavailable testosterone, and free testosterone in an approximate 1% annual decline after age 30. In cross-sectional and longitudinal studies of men aged 30

or 40 years and above, total, bioavailable and free testosterone concentrations fall with increasing age with a steeper decline in bioavailable and free testosterone compared with total testosterone concentrations (Harman *et al.*, 2001; Feldman, H.A, 2002; Liu *et al.*, 2007).

Androgen receptors (AR) are found in brain regions involved in memory and cognition, particularly the hippocampus and the frontal regions. The hippocampus has been studied extensively as it is vulnerable to the course of aging and essential to successful spatial navigation performance in animals and humans (see review Driscoll *et al.*, 2005). The hippocampus is a target structure for gonadal steroids with a relatively high concentration of androgen receptors, suggesting a likely relationship between AR and hippocampus-dependent cognition.

1.4.2 Testosterone synthesis (Steroidogenesis).

The conversion of cholesterol into androgens takes place in Leydig cells. Although Leydig cells are of major importance for the generation of circulating androgenic hormones, the adrenal cortex also contributes to this production. The production of steroids (steroidogenesis) is not limited to endocrine glands only, but in very small amounts can also be produced in brain cells (Baulieu, 1997). Although the contribution of cells in the nervous system to circulating hormone is very small, local production of steroids can be physiologically very important (King *et al.*, 2002) especially when transport and clearance are low.

The initial step in steroidogenesis is the conversion of cholesterol to pregnenolone (PREG) on the inner mitochondrial membrane by the enzyme cytochrome P450 side chain cleavage (P450_{sc}). The amount of P450_{sc} protein can be regulated directly by hormone-dependent phosphorylation. When the amount of P450_{sc} is constant, cholesterol is being transferred from the outer mitochondrial membrane to the inner mitochondrial membrane, which is deficient in cholesterol. And then PREG converted to progesterone (PROG), which is the first biologically important steroid in the pathway, by the enzyme 3 β -hydroxysteroid dehydrogenase-isomerase (3 β -HSD) in the endoplasmic reticulum, or to dihydroepiandrosterone (DHEA) by cytochrome P450c17.

P450c17 is a key branching point in steroid hormone synthesis, directing pregnenolone towards the sex steroids (both hydroxylation and cleavage activities of the enzyme), the glucocorticoids (only hydroxylation) or the mineralocorticoids, if neither of the enzyme activities is participating. The former pathway results in the synthesis of of PROG and PROG metabolites. The latter pathways results in the formation of testosterone via conversion of androstenedione by 17 β -HSD. Testosterone can in turn be converted into estradiol via the enzyme P450 aromatase (Figure 1.4).

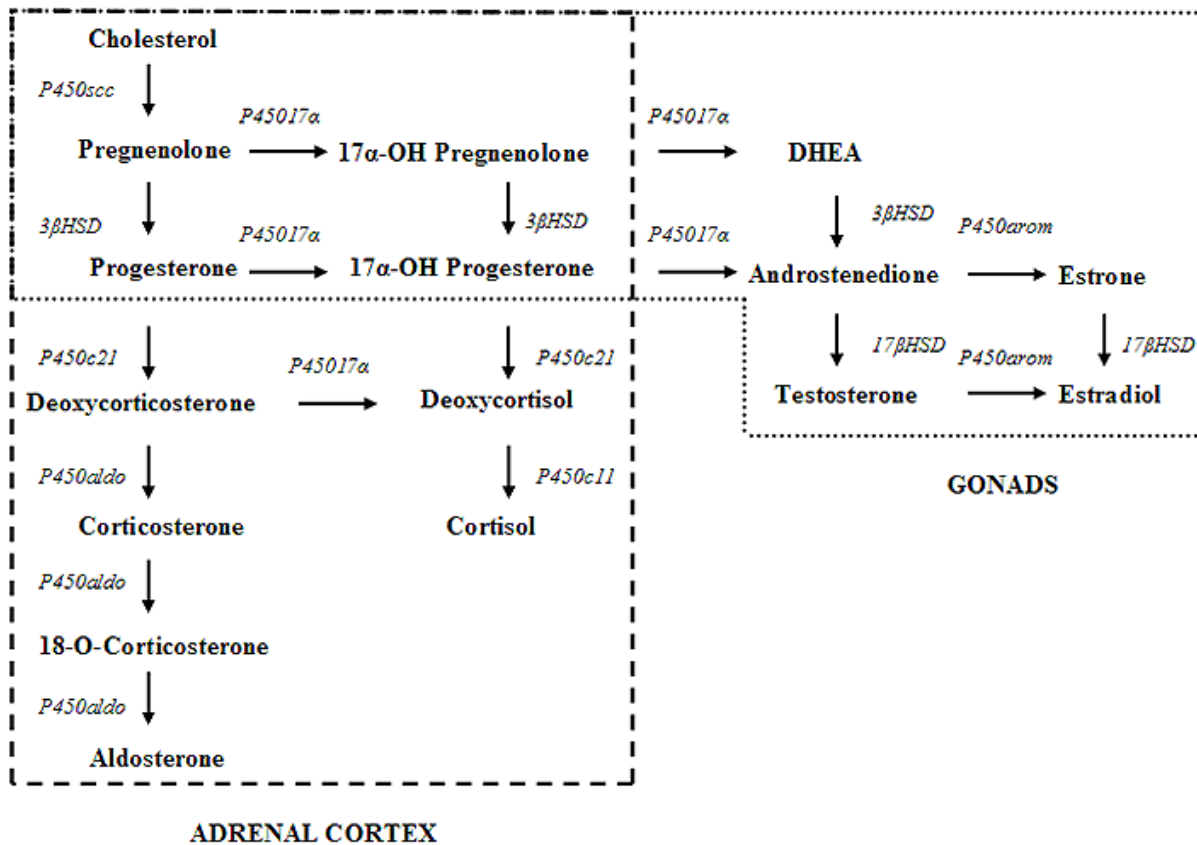


Figure 1.4 : Steroid hormone synthesis pathways in CNS. All steroid hormones are synthesized from cholesterol and the end products can be classified according to their principal effects; mineralocorticoids (aldosterone), glucocorticoids (cortisol in human, corticosterone in rodents), progestins, androgens and estrogens (<http://herkules.oulu.fi/isbn951426844X/html/graphic99.png>).

Brain steroid synthesis proceeds in the following manner. First, glutamate is released from the presynapse induces a Ca²⁺ influx through the NMDA receptors. The Ca²⁺

influx drives STAR or peripheral benzodiazepine receptor (Papadopoulos, 1993) to transport the cholesterol to the mitochondria. The following process of steroidogenesis is similar to that in Leydig cells.

1.4.3 Testosterone and Aging

Age-related changes occur in numerous physiological systems. Research indicates that in both human and non-human animals gonadal function is compromised in aged individuals. Unlike the marked changes in the sex hormones that accompany menopause in females, the analogous process in males, termed andropause or partial androgen deficiency in the aging male (PADAM), is characterized by a subtle and generally gradual decline in circulating testosterone levels. It has been estimated that testosterone levels decrease on average at a rate of 1% yearly after 30 years of age (Rajfer, Jacob, 2003) but with variations between individuals and reflecting their state of health. This reduction in hormone levels has a number of physiological and psychological effects. It can manifest in the aging male as sexual dysfunction, loss of muscle and bone mass, increased frailty, depression, and cognitive impairment (Bates *et al.*, 2005). Eventually, it will lead to an increased risk of developing AD. It has been reported that the primary site of decreasing testosterone in aging is its biosynthesis in Leydig cells (Rajfer, Jacob, 2003). The rate limiting step of testosterone biosynthesis is transfer of the cholesterol to the inner membrane of mitochondria to start the steroidogenic process as mentioned above.

In a recent study by Thilers and colleagues (1995), a significant correlation between circulating testosterone levels and cognitive function in aged man was found. Lower testosterone levels were related to reduced cognitive performance. Low levels of testosterone may also be associated with AD where lower free testosterone levels are observed when compared to age-matched controls (Hogervorst *et al.*, 2004). In addition, blood plasma testosterone levels from a large longitudinal study of men age 32-87 years has shown that low free testosterone levels can be detected 5 years prior to AD diagnosis (Moffat *et al.*, 2004). However, Geerlings *et al.*, 2006 showed that endogenous testosterone levels were not associated with risk for cognitive decline in a group of men aged 71–93 yrs over the 6 years of this latter study.

In order to elucidate the relationship between circulating levels of testosterone and cognition in aging men, the understanding of bioavailable testosterone and total testosterone in plasma or serum was needed. There is a curvilinear relationship observed between sex steroid levels and cognitive functions (see review by Bates *et al.*, 2005). Studies in humans concerning the relationship between endogenous androgen levels and cognitive performance have produced inconsistent results, although there do exist striking sex differences in spatial abilities (Kimura, D., 1996). These inconsistent results could be explained in part by the fact that each study employed different preparations and doses of testosterone, and participants were treated for various durations, and the selection criteria for each target population was different.

The relationships between aging and a decline of sex steroid levels remains to be fully elucidated, particularly when it applies to age-related disorders such as AD and the effects in the brain, an androgen-responsive tissue. Evidence indicates that the brain is a steroidogenic organ, with the ability to synthesize steroid hormones from cholesterol. The steroids produced in the brain and accumulate in the nervous system are termed neurosteroids (Mellon, S.H *et al.*, 2001; Plassart-Schiess E. and Baulieu E.E, 2001). In the brain, testosterone can be metabolized to dihydrotestosterone (DHT) and bind to androgen receptors (AR). Or it can be converted to estradiol by the enzyme aromatase. Both androgen receptor (AR) and aromatase are found in regions of the brain involved in memory and learning, including the hippocampus and amygdala (Janowsky, J.S, 2006; McEwan, I.J, 2004).

Testosterone inhibits generation of A β in the rat brain (Ramsden, M, 2003), reduces A β -induced neurotoxicity in culture (Pike, C.J., 2001), prevents hyperphosphorylation of tau (Papasozomenos, S.C, and Shanavas, A, 2002), and protects against oxidative stress (Ahlbom, E, *et al.*, 2001). Moreover, testosterone supplementation in aged rats reduces glial fibrillary acidic protein (GFAP) expression (Day, J.R., 1998) commonly observed in the aging brain in association with inflammatory cytokine. Gouras and colleagues, 2000 showed that treating cells with testosterone reduced A β secretion by promoting the non-amyloidogenic processing of APP and stimulating the production of α APPs, which has been shown to have neurotrophic and neuroprotective properties. These *in vitro* and *in vivo* studies indicate the beneficial role for testosterone supplementation in

the aging organism and some mechanisms by which testosterone may exert its effects on the brain.

1.4.3.1 Testosterone replacement therapy

The earliest study of hormone replacement therapy (HRT) and Alzheimer's disease was published in 1984 by Heyman *et al.* Previous epidemiological studies concerning gender differences in AD have often resulted in conflicting data (Fratiglioni *et al.*, 1997; Letteneur *et al.*, 1994), yet most studies support the high prevalence and incidence for AD in women (McGonigal *et al.*, 1993 ; Rocca *et al.*, 1991). Because of this gender-specific reason for AD and not to other dementias, research has been going on in focusing on the role of sex steroids estrogen and testosterone in the pathogenesis of the disease. In contrast to the considerable work done on estrogen replacement therapy (ERT), clinical trials with testosterone replacement therapy (TRT) for AD have been relatively scarce and these studies involved small samples of participants.

The relationships between aging and lower testosterone levels have led to increased interest in the use of TRT with the aim of delaying the aging process. TRT has been shown to be effective and beneficial for andropausal men (Matsumoto, A.M.2002; Matsumoto, A.M.2003; Gruenewald, D.A, Matsumoto, A.M. 2003). In addition TRT has the ability to increase bone density, muscle strength and lean body mass in hypogonadal man (Thilers, PP *et al.*, 2006).It has also been reported that aged man receiving TRT exhibited increased muscle mass and strength and improvements in cognitive performance (reviewed in Cherrier, MM, 200; Morley, J.E., 2000; Tenover, J.S., 1992). Furthermore, an emerging area of research demonstrates that low levels of androgen may play a role in the onset and progression of AD (Rosario, E.R *et al.*, 2004; Moffat, S.D *et.al.*, 2004).

Despite nearly a half century of research on aging and sex steroids in men, answers to key questions that would allow us to confidently assess risk to benefit ratios for testosterone replacement in older men with hypogonadism remain uncertain. Although it is now reasonably clear that a significant percentage of healthy older man have decreased testosterone levels consistent with hypogonadism, the clinical implications of

this change remain uncertain. Testosterone replacement therapy which is approved by FDA as a treatment for male hypogonadism, has been found to be effective in ameliorating a number of symptoms associated with low testosterone. However, there have been fewer studies, particularly placebo controlled randomized trials, in population of middle aged or older men who do not meet all the clinical diagnostic criteria. Furthermore, studies of testosterone therapy in older men generally have been of short duration, involving only a small number of participants, and often lacking adequate controls. In its review from the literature Free Executive Summary about testosterone and aging : clinical research directions, 2004, the reviewers identified only 31 placebo-controlled trials of testosterone therapy in older men with the largest sample size involved 108 participants and the duration of therapy in 25 of 31 trials was 6 months or less. There is only one study placebo-controlled trial lasted longer than a year.

Studies that have assessed more specific domains of cognitive function in older men have produced mixed results. In a long-term longitudinal study, 407 elderly men aged 50–91 years at baseline were assessed for cognitive status and testosterone levels. Tests of cognitive function and behaviour included verbal and visual memory, mental status, visuomotor scanning and attention, verbal knowledge/language, visuospatial ability and symptoms of depression. Overall, an increased free testosterone index was associated with improved scores on visual and verbal memory, visuospatial function and visuomotor scanning. There is a strong biological rationale to support the potential protective effects of testosterone against the age-related cognitive decline and the development of dementia in men. Cognitive impairment has been observed in men with prostate cancer undergoing chemical castration, further indicating that testosterone may play an important part in cognitive function. Low serum testosterone levels have been associated with low performance in cognitive tests and at least one large longitudinal study has demonstrated that low testosterone levels are often present up to 10 years prior to the onset of AD. In older healthy men, lower levels of endogenous testosterone may be associated with poor performance on at least some cognitive tests. Lastly, the results of randomized, placebo-controlled studies indicate that testosterone substitution may have moderate positive effects on selective cognitive domains in older men with and without hypogonadism. Similar results have been found in studies conducted in patients with existing AD or MCI.

Animal research demonstrated a link between reduction in testosterone levels, the production of A β , and the extent of this change is influenced by the APOE ϵ 4 genotype. As described above, several studies showed a decline in testosterone correlates with aging in men. Some researchers proposed a relationship between the development of AD and the reductions of testosterone. Should this hypothesis be true, links should exist between AD, aging, cognitive decline, and reduction of testosterone and also between testosterone replacement therapy and cognition in elderly AD men.

It is well known from the animal literature that the effects of testosterone mediated through the androgen receptors are widespread but complex, and as such may have specific effects on certain aspects of cognition. However, studies of exogenous testosterone administration in men have provided mixed results. Also, there have been relatively few studies examining the relationship between sex steroids and cognition in older people. Many of the studies are associative in nature. The possibility remains that low testosterone levels are an outcome of age-related cognitive decline and AD pathology rather than a marker for the disease. It also remains unresolved whether normal levels of testosterone are required for optimal performance on gender and age sensitive tests, and further whether its effects are direct or occur through the conversion of testosterone to dihydrotestosterone or estradiol. Even though most results support the facts that testosterone can enhance cognition in older men, much research is needed to be done before recommendations for clinical practice can be made. The results, although by no means conclusive, indicate a role for testosterone in cognitive processing and suggest a further investigation and replication in larger samples. There are quite a lot of reviews about the attention that testosterone is gaining in cognition and aging research. They all recognize that before we can fully understand the neurocognitive effects of steroid hormones, it is imperative to characterize the neural and cognitive effects of testosterone loss and its subsequent supplementation. Both human and non-human animal studies are needed in order to further our understanding of the genetic and environmental effects as well as the processes that may be influencing variability in behavior and its functional outcome with aging. An important feature of animal models of aging and animal models in general, is that they closely mimic the characteristics of human conditions and that they are sensitive to individual differences. But even more imperative is to keep in mind the lessons learned from the

animal research as we enter the decade where it is important to translating the discovery power of neuroscience to improve the diagnosis and treatment of mental disorders.

There are lessons to be learned not only from the non-human animal studies but also the literature investigating the effects of hormone replacement therapy in women which is much more extensive compared to that available on men. Although the Women's Health Initiative Memory Study (WHIMS) (Shumaker, S.A, 2003) was terminated early as it seemed to contradict the previous promising observations regarding HRT and the overall health risks were unexpectedly found to outweigh the benefits, this now much criticised trial raised awareness regarding the possible role for hormones in aging and cognition. Now that estrogen is no longer seen as the silver bullet for AD and age-related cognitive decline as many had hoped, the focus has shifted somewhat to testosterone and the andropause,. But still we have to be aware that although recent findings from observational studies and small-scale testosterone trials in elderly men are promising, additional studies on a much larger scale are required before any conclusions and recommendations for clinical practice can be reached for the use of testosterone in preventing or ameliorating AD.

Information based on clinical trials as mentioned above has added to evidence for benefits and side effects of testosterone replacement in hypogonadal patients and animal models. In general, most researchers agree with short term safety of testosterone replacement therapy but long term safety is still unknown. In this regard, larger long-term studies are needed. It has been estimated that approximately 5000-10000 men need to be randomized and treated for 5-7 years to assess long term safety that if found to be successful would serve as a general recommendation for all men (Bhasin, S., Buckwalter, J.G, 2001)

The discussion above outlines the importance of reductions in testosterone in AD risk and pathogenesis and the potential of testosterone in ameliorating the cognitive decline and neurodegeneration in AD. However, evidence from the original findings of Bowen *et al.*, 2000 and Short *et al.*, 2001 and subsequent findings from our lab and others have, implicated another hormone within the HPG axis, luteinizing hormone (LH), in AD risk and pathogenesis. Below, I review the literature providing evidence implicating LH in

AD risk, cognitive impairment and modulating Ab levels and discuss lowering LH levels as a potential therapeutic strategy for AD.

1.4.4 Regulation of testosterone by LH

The hormones of the hypothalamic–pituitary–gonadal axis include gonadotropin-releasing hormone, luteinizing hormone (LH), follicle-stimulating hormone, estrogen, progesterone, testosterone, activin, inhibin, and follistatin. Each of these hormones is involved in regulating reproductive function by participating in a complex feedback loop that is initiated by the hypothalamic secretion of gonadotropin-releasing hormone (Genazzani *et al.*, 1992). LH and its receptor are present in increased quantities in brain regions susceptible to degeneration in AD with the most abundant in hippocampus and known to cross the blood-brain barrier (Bowen R.L, Smith M.A, Harris P.L, *et al.*, 2002). LH is also known to be mitogenic, and could therefore initiate the cell cycle abnormalities known to be present in AD-affected neurons. Therefore, it is thought that LH plays a role in the pathology of AD. In this regard, there are epidemiological data supporting this notion. Paralleling the female predominance for developing AD (Rocca, *et al.*, 1991; Jorm, *et al.*, 1987), LH levels are significantly higher in females than males (Zandi *et al.*, 2002), in post-menopausal women, and are still higher in individuals who have AD (Short *et al.*, 2001). Like epidemiological data, experimental data also indicates a role for LH in AD. In this regards, experiment in cell culture showed that LH increases amyloidogenic processing of amyloid beta protein precursor (Bowen *et al.*, 2004), and in animal models of AD, pharmacologic suppression of LH and FSH reduces plaque formation, a selective GnRH agonist (leuprolide acetate) in A β PP transgenic mouse has been shown to reduce LH to undetectable levels compared to aged matched controls (Casadesus *et al.*, 2006). Given the evidence supporting a pathogenic role for LH in AD, a trial of leuprolide acetate, which suppresses LH release, has been initiated in patients. A recently completed phase II clinical trial shows stabilization in cognitive decline in a subgroup of AD patients (<http://clinicaltrials.gov/ct/show/nct00076440?orden=6>). These promising findings support the importance of LH in AD and gives way for an alternative therapeutic approach to target this insidious disease.

LH and FSH are required for the development and maintenance of testicular functions. LH is the most important hormone for control of Leydig cell functions and number. LH acts on Leydig cells via LH receptors. The importance of LH receptor and its functional properties has been learned from many studies dealing with receptor's mutation (see review by Themmen and Huhtaniemi 2000 , Ascoli *et al.*, 2002).

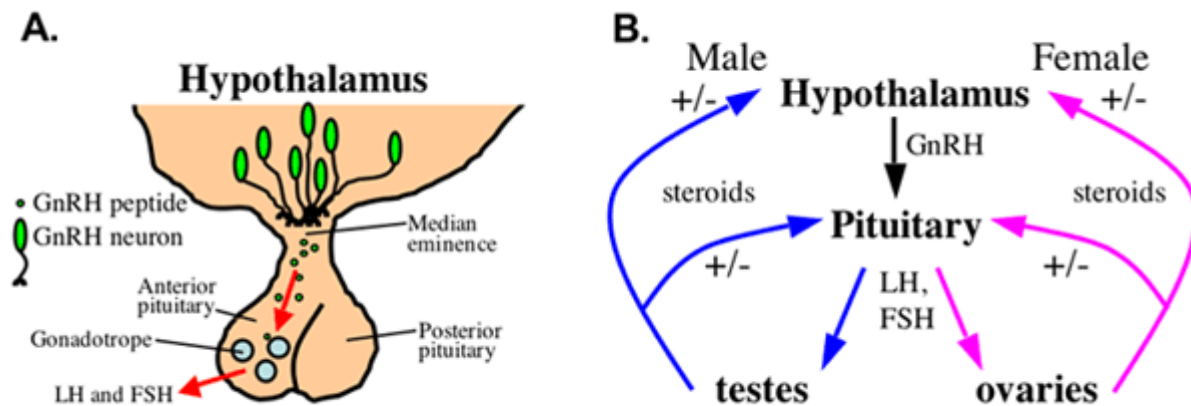


Figure 1.5 : GnRH neurons and the hypothalamic-pituitary-gonadal axis. (A) Gonadotropin releasing hormone (GnRH) are discharged from hypothalamic central nervous system to stimulate the function of gonadotrophs in pituitary gland. (B) The HPG axis. In response to gonadotropins (FSH, LH) the gonads synthesize and secrete sex steroids (<http://www.cellscience.com/Reviews5/Nunemaker5.gif>).

Epidemiologic and biochemical evidence suggests that dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis with menopause/andropause may be the common mechanism driving degenerative changes in the aging brain. This dysregulation leads to the decline in the production of sex steroids and inhibin. The subsequent loss of negative feedback by these hormones on the hypothalamus and pituitary leads to increases in serum activins, increased release of hypothalamic gonadotropin releasing hormone (GnRH), and increased synthesis and secretion of gonadotropins. The increased secretion of GnRH that results from increased activin signaling with the loss of gonadal inhibin (Bilezikjian, *et al.*, 2004) and the loss of

negative feedback by the sex steroids results in a 3- to 4-fold and a 4- to 18-fold increase in the concentrations of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH), respectively, in women. Likewise, men also experience a greater than 2-fold, and 3-fold, increase in LH and FSH (Bowen RL, Atwood CS. 2004).

Experimental studies for the loss of sex steroids in promoting Alzheimer's disease has come primarily from studies demonstrating an increase in A β deposition following menopause/andropause and ovariectomy/castration which suppress the sex steroid concentration, and by the influence of sex steroids to the modulation of A β PP processing and A β generation *in vitro*. Testosterone have also been shown to alter neuronal A β PP processing toward the non-amyloidogenic pathway both *in vitro* and *in vivo*. Testosterone treatment with 200nM to 2000 nM of mouse neuroblastoma cells and rat primary cerebrocortical neurons increased secretion of sA β PPa and decreased the secretion of A β (Gouras GK, Xu H, Gross RS, *et al.* 2000). The same result was also observed in human cell lines and primary cultures of rat and human embryonic cerebrocortical neurons (Petanceska *et al.*, 2000; Chang *et al.*, 1997).

Recent study by Tianbing Liu *et al.*, 2007 tested whether LH modulate steroid synthesis in the brain. The result of this study is LH induces neuronal pregnenolone production by modulating the expression of the LH receptor (suppression of serum LH in young rats treated with leuprolide acetate for 4 months increased LH receptor in the brain), increasing mitochondrial cholesterol transport (increased in the expression of steroidogenic acute regulatory protein / STAR), and increasing P450_{scc}-mediated cleavage of cholesterol for pregnenolone synthesis. Other hormonal changes in aging males report a small increase in LH until age 70 and then increases significantly partly due to the reduced responsiveness to GnRH down-regulation. These changes result in the loss of the diurnal rhythm variation of testosterone secretion (Morely, J.E, Kaiser, F.E *et al.*, 1997 ; Lamberts, S.W *et al.*, 1997). Overall studies suggest that in addition to sex steroids, LH also modulates A β generation. Taken together the studies mentioned above offer significant evidence to indicate a role for LH in neurosteroidogenesis, cognitive functioning, and modulating A β production. Thus, lowering LH levels using the GnRH analogue, leuprolide, has been evaluated as therapeutic strategies for AD.

1.5 GnRH agonist and antagonists as a therapeutic strategy for AD.

1.5.1 GnRH

The neuropeptide gonadotropin-releasing hormone (GnRH, also referred to as luteinizing hormone releasing hormone, LHRH,) serves as both a hormone and a neurotransmitter, and it has multiple actions on reproductive physiology and behaviour. At least seven different molecular forms of GnRH have evolved, and nearly all vertebrates studied express at least two different forms of GnRH: chicken GnRH II, and a second form that varies across classes (Muske, E Linda, 1993). GnRH is a small decapeptide that plays a role in the connection between the neural and endocrine systems. This oligopeptide is synthesized and stored in the medial basal hypothalamus. It acts on anterior pituitary gonadotrope cells, which express GnRH receptors, to signal both the synthesis and secretion of gonadotropin hormones : luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the circulation (Conn, P.M, 1994).

1.5.2 Leuprolide and its effect on LH

After the discovery of GnRH in 1971, analogs that have agonistic and antagonistic effects have been used widely in experimental and clinical research. In men, GnRH analogs are the most frequently used treatment for locally advanced or metastatic prostate cancer (Frydenberg *et al.*, 2000) . Leuprolide acetate is the most used GnRH agonist compared to the other GnRH agonists such as goserelin, nafarelin, triptorelin, histrelin, buserelin, and deslorelin (www.leaddiscovery.co.uk). It is a potent suppressor of Luteinizing Hormone (LH) (Ferin, M. *et al*, 1984; Ravivarapu, Moyer and Dunn, 2000) and preliminary data has indicated that it may offer therapeutic benefits against AD.

Leuprolide or leuprolide acetate is a synthetic gonadotropin-releasing hormone agonist (GnRH agonist) that has been designed to mimic the actions of GnRH to improve the cognitive function and slow the progression of Alzheimer's disease (AD). It is a potent suppressor of Luteinizing Hormone (LH) (Ferin, M. *et al*, 1984; Ravivarapu, Moyer and Dunn, 2000).

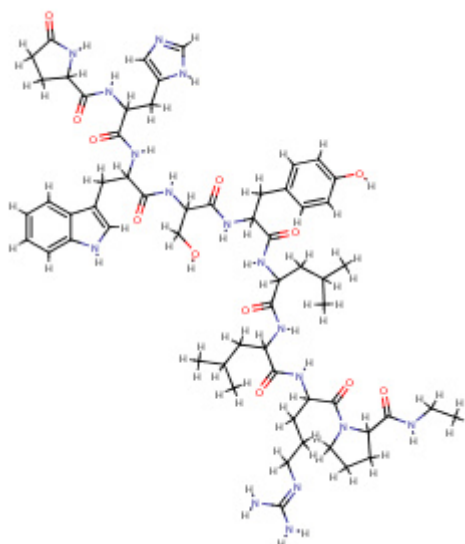


Figure 1.6 : Chemical structure of leuprolide

(www.drugs.com/ingredient/leuprolide.html)

In order for suppression of LH to be successful leuprolide needs to be administered continuously at a sustained level. Mode of action of this drug is by causing constant stimulation of the pituitary GnRH receptors. Short term exposure to leuprolide stimulates production of LH resulting in increased testicular androgen production. However long term continuous administration of leuprolide will soon decrease pituitary secretion (down-regulation) of luteinizing hormone (LH) and desensitization of the pituitary – gonadal axis, which will be followed by suppression of sex hormone production (Chrisp and Sorkin, 1991, Parmar *et al.*, 1990) . Adequate desensitization is usually achieved when there is no significant difference between basal and peak LH levels in response to exogenous GnRH. This agonist analog is more potent than natural GnRH and appears to be capable of occupying pituitary GnRH receptors. This results in a "down-regulation" of receptor activity and gonadotropin release, ultimately decreasing serum testosterone levels to those seen following castration. Numerous studies have demonstrated the effect of leuprolide on LH suppression. Like other GnRH agonists, leuprolide is commonly used to treat hormone related conditions such as prostate cancer, breast cancer, and estrogen-dependent conditions (endometriosis and uterine fibroids) (Wilson, A.C *et al.*, 2007).

Bowen *et al.*, 2004 showed that LH modulates the processing of A β PP and the generation of A β . However in castrated or ovariectomised animals following treatment with leuprolide acetate decreased brain A β was not observed. (Xu, *et al.*, 1998; Gouras, G.K *et al.*, 1998). On the other hand suppression of gonadotropins with leuprolide acetate has been shown to improve cognitive performance and decreases A β deposition in transgenic mice carrying the A β PP Swedish mutation (Casadesus, G, Ogawa, O, Bowen, R.L, *et al.*, 2003). This results shows us that treatment with leuprolide acetate effectively lowered serum levels of the gonadotropins, luteinizing hormone and follicle-stimulating hormone, through gonadotropin releasing hormone receptor desensitization.. Reduction in the levels of these gonadotropins resulted in a 3.5- and 1.5-fold reduction in total brain A β 40 and A β 42 concentrations, respectively, in C57Bl/6J mice (Bowen *et al.*, 2004).

Phase II clinical trials conducted by Voyager Pharmaceutical Corporation leuprolide treatment showed stabilization in cognitive decline in a subgroup of AD patients.. (ALADDIN Study - Phase III, 2007). Basically, female AD patients treated with high doses of leuprolide acetate showed stabilization in cognitive function and activities of daily living (<http://www.secinfo.com/d14D5a.z6483.htm>, pages 56-64). Based on the above findings leuprolide acetate is strong candidate for the treatment of AD.

1.5.3 Naltrexone and its effects on LH

Naltrexone also is a suppressor of LH. However, unlike leuprolide, it is a pure opioid antagonist which has a high affinity for opiate receptor sites, that is used for blocking effects of opioid agonist promptly and completely (Gauthier and France, 1999), This receptor antagonism varies between receptor types with the μ -type receptors being most selectively antagonized as measured by PET (Lee *et al.*, 1988). Since naltrexone has high receptor affinity, it blocks virtually all of the effects of opioids such as heroin. The two opioid antagonists most widely used are naltrexone and naloxone. Naltrexone has far greater therapeutic usefulness than the other opioid antagonist naloxone. First, unlike naloxone which is administered intravenously, naltrexone retains much of its efficacy when administered orally. Secondly, the duration of action of naltrexone is more than 24 hours after moderate doses, unlike naloxone which has a relatively short half-life (Martin *et al.*, 1973).

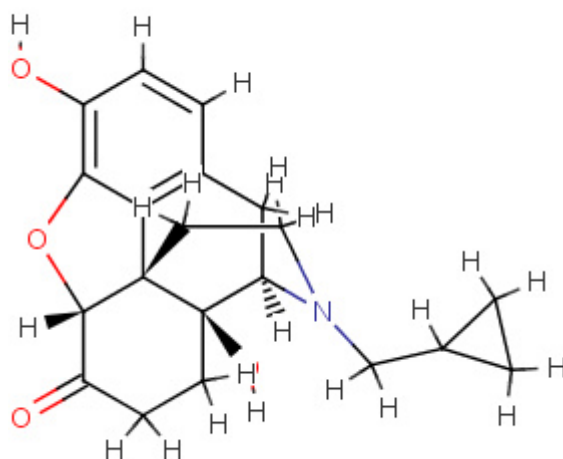


Figure 1.7 : Chemical structure of Naltrexone

(www.drugs.com/ingredient/naltrexone.html)

An opioid is a chemical that works by binding to opioid receptors, which are found principally in the central nervous system and the gastrointestinal tract. Opioid peptides are found throughout the central nervous system, and have profound effects on neuro-endocrine function. The widespread occurrence of opioid peptides and their receptors in brain and the periphery correlates with a variety of actions elicited by opioid agonists and antagonists on hormone secretion. Interestingly opioid receptor antagonists can influence attention and memory. Furthermore, according to study by Zubieta, J.K and Roberts E, 1999, it has been hypothesised to be potential therapeutic value in Alzheimer's Disease.

Narcotic drugs and endogenous opiate peptides inhibit the production of luteinizing hormone (LH) by the hypothalamic-pituitary axis in the male and female of several mammalian species (Azizi, *et al.*, 1973; Mirin, *et al.*, 1976 ; Cicero, T.J *et al.*, 1976; Van Vugt, *et al.*, 1983; Grossman *et al.*, 1981). The administration of opiate-receptor antagonists alone significantly increase the pulsatile mode of LH release, with increases in the frequency and peak amplitude of both immunoactive and biologically active LH pulses (Veldhuis *et al.*, 1983; Delitala *et al.*, 1983; Ellingboe, J *et al.*, 1982). In addition, the negative feedback actions of androgen and estrogen can selectively

influence either the frequency or the amplitude of the LH pulse. However, whether there is any relationship, between these inhibitory actions of sex steroid hormones and the suppressive effects of endogenous opiates is not known. Recent investigations in the rat have suggested that endogenous opiates may participate in testosterone and estrogen's suppressive effects on LH secretion (Van Vugt, *et al.*, 1983; Leadem, C.A. *et al.*, 1985 ; Cicero, T.J *et al.*, 1979). However, whether functional coupling between these two major inhibitory systems exists in man and is integrated specifically via mechanisms that control one or more distinct properties of pulsatile LH release has not been elucidated.

Several clinical studies of naltrexone effects in women showed that treating women with naltrexone restores menstrual cycles in amenorrheic and/or anovulatory women with high serum LH levels (Lanzone *et al.*, 1993; Genazzani *et al.*, 1995., Remorgida *et al.*, 1990; Wildt *et al.*, 1993; Armeanu *et al.*, 1993; Couzinet *et al.*, 1995) . In addition, long term naltrexone treatment in females with polycystic ovary syndrome (PCOS) has been shown to reduce the amount of LH released. Furthermore the BMI in obese PCOS women were reduced (Fruzzetti *et al.*, 2002), together with insulin levels (Fulghesu *et al.*, 1998; Villa *et al.*, 1997).

Naltrexone also has been used in a small studies to evaluate its potential therapeutic benefits for AD (Hyman *et al.*, 1985; Pomara *et al.*, 1985; Knopman *et al.*, 1986) although with an inconclusive results. However, the major limitations of these earlier studies are the very small sample sizes, in each study with 10 or less dementia patients, and particularly the short duration of treatment which was usually administered for 2-3 weeks. Therefore, in this study, Naltrexone will be used in order to mimic situations of low levels of LH in an *in vivo* model which will allow us to assess the effect of LH levels and testosterone hormone therapy on A β production.

1.6 Role of Glia in Hormonal Signalling

Glial cells are non-neuronal cells that provide support and nutrition, maintain homeostasis, form myelin, and participate in signal transmission in the nervous system.

Glial cells are classified in two main groups : microglia and macroglia. Microglia is macrophage-like cells that play important roles in responses of the brain to injury or infection. Macroglia are subdivided in four specialized cell types : ependymal cells, Schwann cells, oligodendroglia, and astroglia. Of these cell types astroglia exhibit GFAP expression (see review Luis, M., Garcia-Segura, 2004)

Over the last several decades research has focused largely on the effects of hormones on neuronal metabolism, with relatively little attention given to glial cells. Glial cells greatly outnumber neurons in the brain, they express many of the genes linked to AD. Together with neuronal cells they share expression of a number of hormone receptors. These include receptors for melatonin, thyroid, vasopressin, oxytocins, leptin, and steroid hormones and likely targets for steroid hormones (Prevot, 2002), and are subjected to many of the same environmental conditions as are neurons. Studies of AD patients and of animal and cell culture models of AD have demonstrated altered glial expression of a number of AD associated proteins. It is thought that A β plays a role in inducing many of these alterations (McGeer *et al.*, 2002).

In AD, activated microglia congregate around the amyloid plaques and degenerating neurons and may produce toxins and inflammatory cytokines that contribute to neurodegenerative processes (review by Mattson, M.P, 2004). In response to cytokines or other hormones these glial cells produce a number of proteins particularly glial fibrillary acidic protein as well as steroid hormones. For example, the expression of glial GFAP is strongly regulated by gonadal hormones in brain areas including the hypothalamus and hippocampus, and the promoter region of GFAP gene contains hormone responsive elements (HRE) (see overview Cynthia L.Jordan, 1999). Finlay and Kritzer, 1999 presented the first *in vivo* evidence that glia express androgen receptors by examining the cortices of adult rhesus monkeys. Several studies also has demonstrated that glial cells express steroid and thyroid hormone receptors (Barakat-Walter, *et al.*, 1999; Gudino-Cabrera and Nieto-Sampedro, 1999; Garcia-Segura, *et al.*, 1999; Vardimon, *et al.*, 1999). Given the evidence that glia in the brain express steroid receptors, steroid could potentially alter neuronal function by exclusively regulating a variety of glial mechanisms.

Integration of hormonal signalling by glial cells occurs in at least two fundamental ways. The first is that the hormone acts directly on the glia, which in turn acts on the neuron to modulate its function (Garcia-Segura, 1999). The second way is the hormone acts first on the neuron, which then releases a substance to target the glia, which then presumably signals back to that and other neurons (Garcia-Segura, 1994).

Garcia-Segura and colleagues were among the first to show that natural fluctuations in estrogen levels stimulate a coordinated and dramatic reorganization of synapses and glia (Olmos, *et al.*, 1989). For example, in the arcuate nucleus of the hypothalamus, an area that is sexually dimorphic in its synaptic connectivity, the pre-ovulatory surge in estrogen affects glia to increase their GFAP expression. (Garcia-Segura, 1994). As estrogen levels decline, glia withdraw their extended process. Exogenous estrogen produces these same effects in ovariectomized adult females. In addition, estrogen does not induce similar morphological changes in the arcuate nucleus of adult males (Horvath, *et al.*, 1997). However, it is still not clear whether estrogens act directly on glia or neurons to alter synaptic connectivity. Research regarding testosterone effects in glia is still lacking and needs to be explored further.

Several studies show that steroids regulate the reactive state of astrocytes and microglia (Garcia-Segura, *et al.*, 1999; Mor, *et al.*, 1999; Nichols, 1999). As reviewed by Nichols, 1999 activation of astrocytes and microglia is a well-known marker of brain aging. Importantly, glial activation associated with normal aging, can occur in the absence of neuronal degradation. Nichols found that while adrenalectomy induced a marked increase in GFAP gene expression in the brains of non aged rats, adrenalectomy had no effect on GFAP gene expression in aged rats.

In summary, although there is still much to be learned on the role of glial cells in neuroendocrine regulation and hormonal signalling, we know that glial cells are able to respond to hormonal and neuronal signals and can produce local active hormonal metabolites whenever and wherever they are needed under physiological, pathological conditions and their association with aging.

1.7 Summary and project rationale

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive memory loss, impairments in behaviour, language and visuo-spatial skills and is ultimately fatal. In the majority of cases, AD onset occurs after the age of 65 (late onset) and is sporadic in origin. Only approximately 5-10% of cases have a disease onset before the age of 65 (early onset). These are often familial forms but only a few that are caused by autosomal dominant mutations in certain AD linked genes. One of the major pathological hallmarks of AD is the presence of neuritic plaques in the brain. These plaques are formed from the build-up of a peptide known as beta-amyloid ($A\beta$). There is now evidence to show that sex hormones play an important role in reducing $A\beta$ levels and so has the potential to protect individuals against developing the disease.

Our laboratory was the first to demonstrate clinically that a reduction in testosterone levels following chemical castration resulted in increased plasma beta $A\beta$ levels and subsequently showed that in elderly men a decrease in testosterone levels was associated with increased plasma $A\beta$ levels. However, the question remains whether changes in plasma testosterone result in changes in $A\beta$ in the CSF and the brain. In addition, recent research has shown that high levels of the gonadotropin, Luteinizing Hormone (LH), are associated with AD and modulate $A\beta$ production. However, the relative contribution of LH and testosterone in the pathogenesis of AD remains to be elucidated.

In order to determine whether testosterone modulates $A\beta$ in the brain I have investigated the effects of its removal and replacement on the levels of this peptide in castrated male guinea pigs. LH was also investigated in this model by either its chemical suppression and through implants.

The efficacy of testosterone on cognition in high risk men by employing a randomized placebo controlled double blind study has been assessed and correlated with a number of key blood and brain biomarkers. These findings will serve as the basis for the establishment of a larger, multicentre trial for the prevention of AD.

1.7.1 Significance of the Study

This study will extend our understanding of the role of testosterone on A β metabolism in an animal model. It will also investigate the potential roles of testosterone and LH on A β metabolism and AD pathogenesis in a human clinical trial. The outcome of this study has the potential to influence the development of an effective approach for the prevention and/or treatment for AD. While there is a high incidence of AD in Indonesia, which is double the incidence of AD in Australia, no clinical trials have been conducted on AD in the Indonesian population to date. Therefore, the current project will directly benefit Indonesia as it will investigate the role of testosterone in the local population. Furthermore the findings generated has the potential of benefitting the global community world-wide.

1.7.2 Hypotheses

- LH increases brain A β levels in guinea pigs
- Testosterone regulates blood and brain A β levels in guinea pigs
- Suppression of LH and testosterone decrease brain A β levels in guinea pigs
- Testosterone treatment in men reduces plasma A β levels
- Testosterone treatment in men improves cognition *in vivo*
- Testosterone treatment in men alters brain metabolites
- Efficacy of Testosterone treatment in men is determined by baseline hippocampal volume.

1.7.3 Objectives

- To investigate whether testosterone regulates blood and brain A β levels in guinea pigs
- To investigate whether LH decreases brain A β levels in guinea pigs

- To investigate whether suppression of LH and testosterone supplementation alter brain A β levels
- To investigate whether testosterone treatment in men reduces plasma A β levels
- To investigate whether testosterone treatment in men improves cognition *in vivo*
- To investigate whether testosterone treatment in men alters brain metabolites
- To investigate whether hippocampal volume determines efficacy of testosterone treatment in men.

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Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Participants

The clinical study was undertaken at the Siloam Hospital in Lippo Karawaci, Tangerang, Indonesia. Human ethics approval was obtained from the Independent Human Ethics Committee, Faculty of Medicine from the University of Indonesia as well as Edith Cowan University, Western Australia prior to commencement of the study. The participants were recruited from all areas surrounding Jakarta and Tangerang, Indonesia, including patients from Siloam Hospital, Lippo Karawaci, Indonesia and the nursing homes from those areas. All participants were screened based on the eligibility criteria (details on Chapter 2 Section 2.1.1.1) prior to the commencement of the study. Only male subjects who had provided written informed consent and met the eligibility criteria were included in either short term study or the long term double blind randomized placebo-control clinical trial.

2.1.1.1 Eligibility Criteria

The following eligibility criteria were used: 1) Male 50 years of age or older; 2) Subjects were selected based on their MMSE assessment. A score of 24 or above determined eligibility of inclusion in the trial. Final decision of inclusion was determined jointly by a neurologist, neuropsychologist, and the researchers in charge of the project; 3) Participants must have a testosterone level of less than 300 ng/dL (~10.4 nmol/L). 4) must have normal range of prostate-specific antigen (PSA), 5) Blood pressure must be within normal limits) must not have diabetes mellitus, 7) must have normal liver and kidney enzyme function and 8) must have not suffered from major head injury. The screening test, included a testosterone measurement, Mini Mental State Examination (MMSE), and clinical interview about subject's medical record (i.e. whether they have had any medication such as donepezil or rivastigmine), were also carried out to decide the eligibility of the subjects. All participants in this study had at least 6 years of education.

2.1.1.2 Biochemistry Laboratory Tests

2.1.1.2.1 Collection of Samples

Blood samples collected from participants at each visit using venipuncture underwent a full blood count performed by the diagnostic laboratory of Siloam Hospital, Karawaci.. Blood samples (\pm 45 ml) were collected using serum separating tubes (SST) that include a gel and blood clot activator), EDTA, and heparin collection tubes. Blood in SST collection tubes were centrifuged at 2500 rpm for 10 minutes to separate the serum. The serum was removed, then aliquoted \sim 1ml to which 10 μ l (Xug/ml) of protein inhibitor was added (aprotinin - Sigma) was added. Blood in EDTA and heparin collection tubes were centrifuged at 1000 rpm for 10 minutes at RT, to separate the plasma, leucocytes, and blood. Plasma was removed and further centrifuged at 2500 rpm for 10 minutes to harvest platelets. The resulting supernatant (plasma) was aliquoted \sim 1-5 ml and 10 μ l protein inhibitor (aprotinin - Sigma) was added to each ml of plasma.

The platelets were washed in 5 ml of saline and then centrifuged at 2500 rpm for 10 minutes, to remove saline. The platelets were resuspended in 200 – 250 μ l of saline prior to aliquoting to a fresh tube.

For harvesting leucocytes, whole blood was centrifuged at 2200 rpm for 5 minutes, the leucocytes added to another tube with 3-4 ml ice cold Ficoll and centrifuged again at 1100 rpm for 15 minutes. The Ficoll was removed and leucocytes were washed with 10 ml of saline (0,9% NaCl) and centrifuged at 2500 rpm for 10 minutes. This step was repeated to remove residue Ficoll or plasma . Leucocytes were preserved in 1ml Fetal Bovine Serum (FBS – Invitrogen) containing 10% Dimethyl sulfoxide (DMSO - Sigma) for APOE genotyping. All blood products were stored at -80°C prior to shipment to Perth. Leucocytes were collected for APOE genotyping (see below section 2.1.1.2.2)

2.1.1.2.2 Blood Biochemistry

Serum samples underwent a full blood biochemistry at the Path West Diagnostic laboratories (Perth, Western Australia) to be measured of the following hormones : LH, estradiol, and insulin, SHBG, albumin, glucose, and PSA levels were also measured.

Lipid profile such as HDL, LDL, triglyceride, and cholesterol levels were also performed in plasma heparin samples. Testosterone and DHT levels were measured in serum samples by gas chromatography-mass spectrometry (GC-MS) at the ANZAC Research Institute, Sydney. Plasma and CSF underwent analysis for A β 42 and A β 40 levels using ELISA as described in section 2.2.5 and was performed in Professor Martins laboratory.

2.1.1.2.3 APOE genotyping

All the leucocytes were thawed and spun at 4,000 rpm for 3 minutes to separate the leucocytes pellet from FBS mixture. The pellet was washed in 1ml 0.9% NaCl and spun at 4,000 rpm for 3 minutes to remove all the excess FBS mixture. The washing step was repeated twice in order to get a clear pellet. The pellet was gently resuspended in 550 μ l TES buffer (10mM Tris-HCl, 100mM NaCl, 1mM EDTA, pH 8), then added 60 μ l of 20% (w/v) SDS and 20 μ l of proteinase-K. The mixture was incubated overnight with movement in a 37°C incubator. An equal volume of phenol (~600 μ l) was then added into the mixture and mixed. An addition of phenol resulted in cloudy mixture. After centrifugation at 13,000 rpm for 8 minutes, the mixture was separated into 3 layers, i.e. (from top to bottom): DNA layer, phenol and cell debris at the bottom of the tube. This phenol purification step was repeated with 300 μ l phenol to ensure the purification of DNA from protein and cellular debris. DNA was transferred into a clean DNase-free tube with 50 μ l of 3M sodium acetate (NaC₂H₃O₂) and 1ml of 100% alcohol (cold) was added to the DNA. The DNA mixture was then gently shaken until the white DNA thread is visible. After centrifugation at 13,000 rpm for 15-25 minutes. A DNA pellet appeared on the bottom of the tube and the supernatant was discarded. One ml of cold 100% alcohol was then added to the pellet and centrifuged at 13,000rpm for 5 minutes. The DNA pellet was then washed with 1ml cold 70% ethanol and spun at 13,000 rpm for 5 minutes. The excess 70% ethanol was discarded and the DNA was air-dried for 5 minutes to totally remove any residual ethanol. DNA-free water (200-300 μ l) was then used to resuspend the DNA pellet prior to APOE genotyping. DNA concentration was measured using Nanodrop which requires sub-microlitre volumes for assessment at 260nm and 280nm.

APOE genotyping were determined by observing migration patterns of DNA on polyacrylamide gels from each individual following PCR. 1.1µl DNA was mixed with 14µl of the master mix contains the following:

Solution	1 reaction (µl)
DMSO	1.5
10x polymerase buffer	1.5
2mM dNTPs	1.5
MgCl ₂	1.2
Primer 1 (5' – TCCAAGGAGCTGCAGGCGGCGCA – 3')	1.2
Primer 2 (5' – GAATTCGCCCCGGCCTGGTACACTGCCA – 3')	1.5
AmpliTAQ gold	0.075
ddH ₂ O	5.35

One drop of paraffin oil was then added on the top of the mixture to avoid evaporation during PCR (polymerase chain reaction). The mixture was then amplified using PCR cycle. The first cycle was the initial denaturation (1X), i.e. 94°C – 5min, 65°C – 30sec, 70°C – 90sec. The cycle was then continued with 35 repetitive cycles, i.e. 94°C – 30sec, 65°C – 30sec, 70°C – 90sec and finished with the chase cycle (1X), i.e. 94°C – 30sec, 65°C – 30sec, 70°C – 10min, 4°C – 1min. The PCR products were then digested using HhaI enzyme and resulted in restriction fragments which were then separated based on their length by gel electrophoresis. The process is known as restriction fragment length polymorphism (RFLP) genotyping. HhaI enzyme (New England Biolabs) was diluted in NEBuffer4 to 5U/µl. One µl of HhaI enzyme was added into each PCR product and incubated at 37°C overnight. The digested product was then run in the 8% polyacrylamide gel (10ml of 8% acrylamide mixture, 20µl of 25% Ammonium persulfate – APS, and 15µl TEMED). Ten µl of samples were mixed with 2µl loading

buffer (1.5gram Ficoll, 0.02gram bromophenol blue, 0.02gram xylene cyanol in 10ml TBE buffer) prior to loading. For the DNA marker, 10µl of DNA-free water was mixed with 2µl PUC19 DNA marker and 2µl loading buffer. The gels were then electrophoresed at 110 Volt for 1.5 hours using TBE buffer (10.8gram Tris-HCl pH 8.3, 5.5gram boric acid, 0.94gram Ethylenediaminetetraacetic acid (EDTA) in 1 litre of ddH₂O. The gels were washed in 50ml ddH₂O with 1µl etidium bromide (EtBr) with shaking for 5 minutes. Three washing steps were followed using 50ml ddH₂O. The visualization of the APOE bands was performed under GelDoc BioRad system.

2.1.1.3 Neuropsychological Testing

In the neuropsychological component of the study, the matched groups of participants were assessed at three different time points :1) baseline and treatment period (week 0-24) 2) washout period (week 24-28) 3) cross-over period (week 28-52), as shown in figure 2.2. The following neuropsychological assessments were implemented: 1) MMSE, a simple test to screen for mental impairment for 5-10 minutes , 2) the Rey Auditory Verbal Learning Test (RAVLT) for 35-45 minutes_. The application in this study of parallel forms of the RAVLT (matched for equal difficulty) ensured that retesting could be conducted over time without the memory data being potentially confounded by practice effects. In addition to these cognitive assessment protocols, a questionnaire to self-assess the health status of the participant (SF36) was also applied. The three related negative emotional states of depression, anxiety and stress were assessed using Geriatric Depression Scale (GDS) and Depression, Anxiety and Stress Scales (DASS) questionnaires. These questionnaires provided information on the emotional states of participants when they underwent the cognitive assessment as depression, anxiety and stress have been known to be associated with cognitive status (Insel, 2002;Jorm, 2000).

2.1.1.4 Brain Imaging

In addition to the medical examination and neuropsychological testing participants also underwent brain imaging which included Magnetic Resonance Imaging (MRI) and Magnetic Resonance Spectroscopy (MRS). MRI was used in the long-term study to

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determine whether the use of testosterone therapy is influenced by hippocampal volume ie individuals with reduced hippocampal volume may obtain less benefit. For the MRI data, we have done the Medial Temporal lobe Atrophy (MTA) scale, starting from rating 0 to 4 in which the higher the scores indicate more atrophy (Korf *et al.*, 2004) measured by three of our experienced radiologist. All of the MRI imaging data (T1,T2) is stored in DICOM format with 7mm thickness slice (TR = 4802, TE=120).

While MRI uses anatomical images to localize regions of interest with high resolution, MRS is a non-invasive, sensitive tool for measuring the concentration of metabolites in the brain, such as N-acetyl aspartate (NAA), Myo-inositol (MI), choline (Cho) and creatine (Cr) levels (Firbank, 2002)..¹H MRS in this study (TR=1500, TE=272) will enhance the diagnostic accuracy of MRI and provide additional information of the brain metabolites changes, such as NAA, Cho and Cr, which can be seen in the form of a graph with specific peaks for each metabolites e.g 2 ppm for NAA (Figure 2.1).

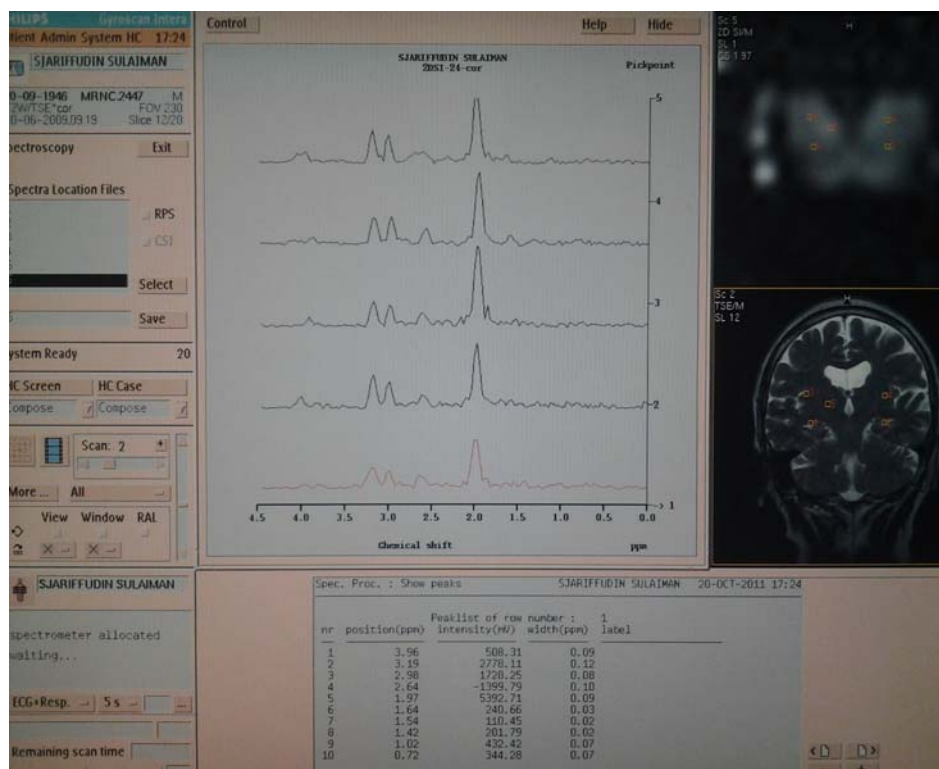


Figure 2.1 ¹H MRS with 5 different spectroscopic points in 1,5T Gyroscan Intera Phillips

We are using two dimensional MR spectroscopy (2D CSI) which enable us to acquire multiple small voxels which gives better information to determine the region of interest (ROI) and the spectroscopic points. We are using 5 spectroscopic points :

- 1) in the right hippocampus
- 2) in the left hippocampus
- 3) in the right medial temporal lobe
- 4) in the left medial temporal lobe
- 5) brain area which have normal spectroscopy

The voxel information from the spectroscopic points can be used to calculate metabolite ratios to creatine-containing compounds. The creatine signal is usually stable over time and therefore is often used as an internal standard to which other metabolites resonance intensities are normalized. Therefore, from this study we would like to determine :

- 1) NAA/Cr ratio (which is found to be lowered in AD)
- 2) Cho/Cr ratio (which some found to be elevated in AD)

MRI and MRS were performed on all participants, with or without testosterone treatment, three times i.e.: at the beginning (baseline), at the end of the initial 24-week treatment period and at the end of the study (see figure 2.2 for the detail), using the Gyroscan Intera 1.5T (Phillip Medical System) at Siloam Hospital Karawaci by the original team of radiographers following strict standardized procedures MRI image acquisition and data handling.

2.1.2 Animal Trial

2.1.2.1 Animals and Treatment Groups

Animal care and experimentation in this study were carried out in accordance with ethical guidelines published by NH&MRC (National Health and Medical Research Council) Statement on Animal Experimentation and approved by the UWA Animal Ethics Committee and Edith Cowan University.

Adult male guinea pigs weighing 500 grams (\pm 6-8 weeks old) were obtained from the BSAU animal care unit at UWA (Perth, WA, Australia). Animals were kept in a 1:1 light and dark cycle room at the BSAU animal care unit with free access to water and a soy-free diet (in order to eliminate any phytoestrogen intake) obtained from Glenforrest Stockfeeders (Perth, WA). The composition of soy-free diet were formulated from casein (300g/kg), as the protein source, sucrose, glucose and starch, as a carbohydrate sources, cellulose (150g/kg) as a fibre source, refined vegetable oils (100g/kg) as lipid source and vitamin, and mineral supplements. As guinea pigs can not produce vitamin C, a supplementation of vitamin C was also added to their water. Prior to experimentation, all the animals were caged for a week in order for them to adapt to the environment.

2.1.2.2 Hormone treatment pellets and the dosage

The treatment groups are outlined in table 2.1. All the treatments given in guinea pigs were in the form of 40-day slow release pellets manufactured by Innovative Research of America, USA. The active ingredients and dosages used in this study are shown in table 2.2.

Table 2.1. The summary of treatment groups

Treatment Group	Administration of hormone pellets	Day of Sacrifice
Testosterone treatment	Peripherally	18 and 36 days post administration of testosterone pellets.
LH treatment	Peripherally and cortical placement - brain	18 and 36 days (peripherally); 15 and 28 days (cortical placement) post administration of LH pellets.

Leuprolide and testosterone treatment	Peripherally	18 and 36 days post administration of leuprolide and testosterone pellets.
Naltrexone and testosterone treatment	Peripherally	18 and 36 days post administration of naltrexone and testosterone pellets.

Table 2.2 A summary of all pellet dosages used in the animal study

Pellets	Dosage
Placebo	20 mg /kg BW/day
Testosterone	2.5 mg /kg BW/day (Low)
	5 mg /kg BW/day (Medium)
	20 mg /kg BW/day (High)
Luteinizing Hormone (LH)	2.25 µg /kg BW/day (~11.25 IU/kg BW/day)
Leuprolide	0.02 mg /kg BW/day
Naltrexone	8 mg /kg BW/day

2.1.3 Consumables

2.1.3.1 Chemicals

All chemicals used in the study, generally, were of the highest purity and purchased from Sigma Chemical Co. (St. Louis, Missouri, USA), BDH Chemicals Australia Pty.

Ltd (Victoria, Australia) or BioRad (Irvine, California, USA) unless otherwise specified.

2.1.3.2 Antibodies

The human-specific APP antibody, C1/6.1, directed against the last 20 residues of APP was kindly provided by Dr. Paul Matthews (Nathan Kline Institute, NY, USA).

The mouse monoclonal antibody WO2 was kindly provided by Professor Colin Masters (University of Melbourne, Vic, Australia). This antibody, is directed against the amino acid sequence 5-8 of the human A β . The 6E10 antibody (Covance) was used as a capture antibody for either A β ₄₀ or A β ₄₂, and recognizes residues of the N-terminus of A β and R208 as detection antibody (kindly provided by Dr. Pankaj Mehta) .

For detecting GFAP (glial fibrillary acidic protein), the mouse monoclonal GFAP antibody (Sigma chemical Co., Missouri, USA) was used. The polyclonal goat apoE antibody (Millipore) was used to detect the apoE levels by western blot. The polyclonal rabbit anti-cytochrome P450 side chain cleavage enzyme (P450scc – Milipore) was used to detect the levels of P450scc neurosteroid enzyme by western blot. The loading control protein, β -actin, was also examined using mouse monoclonal β -actin antibody (Sapphire Bioscience Pty Ltd, Redfern, NSW, Australia).

Two major secondary antibodies were used in this study, a sheep anti-mouse IgG horseradish-peroxidase (HRP) antibody (GE Healthcare, Buckinghamshire, UK) and a polyclonal rabbit anti-goat IgG-HRP antibody (Dako Co, CA, USA).

2.2 Methods

2.2.1 Clinical Study Design

The testosterone used in the clinical study was applied in the form of a cream (Andromen,[®] 5% Forte) , which was obtain from Lawley Pharmaceuticals (Perth, Western Australia).

Prior to commencing the main “long-term” study, a pilot “short term” study was performed to determine the optimal site to apply the testosterone cream.

2.2.1.1 Short-term (Pilot) Human Study

Short-term (Pilot) Human Study 1

Four male participants were recruited for the pilot study .Before admission of this study, the participants were instructed to fast overnight. At admission, the male participants were underwent a medical examination [height, weight, and body mass index (BMI) were measured]. For this study, the participants were recruited and admitted into hospital for a period of 36 hours. The blood sample were drawn at a baseline (time zero), 2, 4, 8, 12 hour prior to the application of testosterone cream. Testosterone (100 mg in the form of Andromen[®] 5% FORTE cream) was then immediately applied via transdermal route (topically). According to the manufacturer’s information, a once off daily dose of 100 mg [4 cm applied to the trunk (side of body above hips)] will increase serum testosterone concentrations in healthy testosterone deficient men into the mid-normal range. The participants were bled again at 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours post application. The level of serum testosterone and LH were measured for all the blood samples taken.

Short-term (Pilot) Human Study 2

As the preliminary result of the first pilot study indicated that application of testosterone cream on the side of the trunk (above hip) had a variable effect on increasing blood testosterone levels, the pilot study was repeated. In this second pilot study, testosterone

(50 mg in the form of Andromen[®] 5% FORTE cream) was applied on the scrotum to maximise the absorption of the testosterone cream on the skin, as this is an area which contains minimal body fat, has thin skin and a good blood supply [insert refs].

The same male participants were instructed to fast overnight and admitted into hospital for a period of 28 hours. At admission in the morning, the participants were underwent a medical examination [height, weight, and body mass index (BMI) were measured] and were bled for a baseline (time zero), and 4 hours later prior to treatment. Testosterone (50 mg in the form of Andromen[®] 5% FORTE cream) was then immediately applied via transdermal route (topically to the scrotum). The participants were bled again at 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours post-application. The level of serum testosterone, SHBG, (Sex Hormone Binding Globulin) albumin and plasma A β levels were measured.

Table 2.3 Comparison between Short-term Human Study 1 and 2

	Pilot Study 1	Pilot Study 2
	Duration of Study (36 hours)	28 hours
Blood taken prior to the application of testosterone	At baseline (time zero), 4, 6, 4, 8, 12 hour prior to the application of testosterone	At baseline (time zero) and 4 hour prior to the application of testosterone
The site for testosterone application	On the trunk (side of body above hips), topically (transdermal route)	On the scrotum, topically (transdermal route)
Blood taken post to the application of testosterone	At 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours post-application	At 2, 4, 6, 8, 10, 12, 16, 20, and 24 hours post-application

2.2.1.2 Long-term Human Study

A randomized, crossover, placebo controlled and double-blind study was undertaken to study the effectiveness of testosterone (a transdermal formulation) treatment in hypogonadal male subjects. Of the potential participants that were screened, 44 eligible males were recruited and assigned to one of the two parallel groups; placebo (n=22) or testosterone (n=22) treatment. Assignment was random using stratification to ensure each arm of the study was balanced, in terms of individual memory performance at baseline. The study design (shown in Fig 2.2) was such that participants received testosterone treatment or placebo for a period of 24 weeks, followed by a washout period of 4 weeks, after which time, the participant groups were “crossed over”, such that the placebo group received testosterone and *vice versa* for a further 24 weeks. For all participants, there were a total of 11 clinic visits to Siloam Hospital, Lippo Karawaci, within the 52-week study period.

During the first visit, the participants’ blood pressure, height, weight, body fat percentage and body mass index (BMI) were checked and blood drawn for a baseline reading of blood counts, chemistries, serum testosterone level, LH and SHBG, DHT (dihydrotestosterone) and estradiol, cholesterol, lipid concentrations and plasma A β levels (as described in section 2.1.1.2.2). The participants’ *APOE* genotype was also determined. The baseline neuropsychological testing, brain imaging (MRI/MRS) and a CSF sample (for those participants who consented) were collected. CSF samples were collected by lumbar puncture (as described below).

The patient was placed in the lateral decubitus position lying on the edge of the bed and facing away from operator. Patient’s position is in a knee-chest position with the neck flexed. The patient’s head is rested on a pillow, so that the entire cranio-spinal axis is parallel to the bed. Then the operator found the posterior iliac crest and palpated the L4 spinous process, and marked the spot. The skin was anaesthetized using 1% lidocaine in the 5 ml syringe. Then a spinal needle was inserted and once the needle was properly positioned, CSF pressure was measured and the CSF samples were collected. When the CSF begins to flow from the needle, the first few drops were discarded. CSF was not

supposed to be aspirated, because a nerve root might be trapped against the needle and risking the possibility of injury. After 3-5 ml CSF were collected, the needle was withdrawn and the puncture site was dressed with a bandage. The patient was rested in bed for a few hours.

For the testosterone treatment group (n=22), testosterone (50 mg in the form of Andromen[®] FORTE cream) was applied daily on the scrotum via transdermal route (topically) for a period of 24 weeks. For the placebo group (n=22) the same amount of the cream base [dl- α tocopherol acetate (vitamin E), without the active ingredient, testosterone] was applied at the same frequency. Since A β produces hydrogen peroxide, which is a toxic free radical, and antioxidants such as vitamin E have been shown to reduce the amount of damage to brain cells by A β brain {Butterfield, 1999 #50}, the placebo group is important to ascertain what effect, if any, the cream base, may have on reducing A β levels and enhancing memory performance. At each of the six remaining clinic visits i.e. at 4, 8, 12, 16, 20 and 24 weeks during the first treatment period, blood pressure, weight, body fat percentage and body mass index (BMI) were checked and blood drawn for blood counts, chemistries, measurement of serum testosterone, LH, SHBG, albumin, PSA, DHT, estradiol, insulin, cholesterol, lipid and glucose concentrations and plasma A β levels, while neuropsychological testing were only included at 8, 16 and 24 weeks. At the end of 24 weeks a second CSF sample was collected (from those participants that consented) and MRI/MRS performed, followed by a 4-week washout period.

During the 4-week washout period, the participants were given an equivalent amount of cream base containing no active ingredients with the same route of administration. They were also clinically examined and assessed neuropsychologically and blood collected for analysis as described above. At the end of the washout period, neuropsychological testing (as described in section 2.1.1.3) was performed. The placebo and testosterone treatment arms of the study were then crossed-over for a further 24-week treatment period. During this period, the participants were examined every eight weeks (three bleeds in total i.e. at weeks 8, 16 and 24 of the cross-over period); blood collected for analysis as described above and neuropsychological testing performed on all occasions. At the end of the 24-week cross-over period (i.e. week 52 overall, the completion of the

trial) a third CSF sample was collected (from those participants that consented) and MRI/MRS performed.

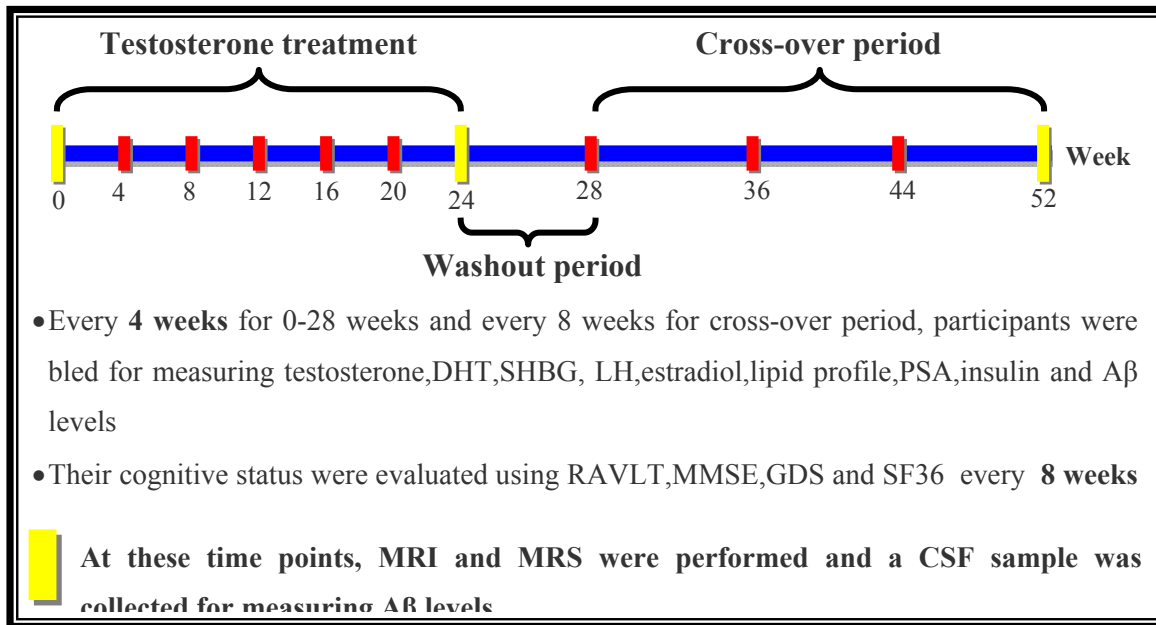


Figure 2.2 : The summary protocol for long-term human study

2.2.2 Animal Trial Design

Guinea pigs were either left intact (non-castrated) or surgically castrated, to mimic the loss of testosterone following andropause in men. All hormone treatments given to the animals were in the form of slow release pellets (Innovative Research of America, USA) and administered either peripherally (subcutaneous insertion into the dorsal neck fat pad of the guinea pig) or cortical placement on the dura above the left and right parietal cortex. The pellets were designed to slowly release the active ingredients over a period of 40 days for the hormone treatments or 50 days for the hormone suppression treatments (leuprolide and naltrexone).

2.2.2.1 Surgical procedures

All surgical procedures used in this study were performed at the surgery room of BSAU animal care unit at UWA (Perth, WA, Australia).

Anaesthesia

All surgical procedures applied to the guinea pigs were performed under halothane anaesthesia by inhalation. Animals were anaesthetized with 3-4% of halothane mix and O₂/N₂ mixture followed by 1-2% of halothane mix in order to maintain anaesthesia during the surgical procedure. However, cortical pellet implantation was performed under atropine-diazepam-hypnorm anaesthesia. Atropine at a dose of 0.05 mg/kg body weight (BW) was given subcutaneously. The 5 mg/kg BW diazepam was then injected intraperitoneally after 5 minutes. After the sedation about 15-20 minutes, the 1 ml/kg BW hypnorm was given intramuscularly. This procedure provided deep anaesthesia for about 45 minutes. If the guinea pigs showed signs of awakening before the surgery was completed, a further 10% of initial dose of diazepam and 25% dose of hypnorm, were given via the same delivery routes. Immediately after the surgery, the analgesic, buprenorphine (0.05 mg/kg BW, subcutaneous) was given and the animals were kept in a warm place to recover.

Surgical castration

Two hundred and thirty five guinea pigs were allocated for surgical castration and weighed prior to the castration. The surgical castration was performed by Dr. David Gardner (veterinary surgeon). Halothane anaesthesia was induced with (3-4% Halothane) and anaesthesia maintained during the surgical castration (1-2% Halothane). All the castrated animals were caged for a week to allow all the endogenous testosterone to be cleared from the body prior to the pellet implantation.

Pellet implantation

Intact or castrated animals were treated with either testosterone, LH, hormone suppression analogues (leuprolide or naltrexone) or a placebo in the form of slow-release pellet (IRA, USA) and administered by the use of two different approaches. The first is peripherally (subcutaneous insertion into the dorsal neck fat pad of the guinea pig) (Wahjoepramono *et al.*, 2008) and the second is cortical placement on the dura above the left and right parietal cortex (Rapisarda *et al.*, 1977; Wahjoepramono *et al.*, 2011). The pellets were designed to slowly release the active ingredient over a period of 40 days for the hormone treatment or 50 days for the hormone suppression treatments. A summary of the experimental treatments is provided in Table 2.4.

For peripheral insertions, smaller pellets were administered subcutaneously using a stainless steel reusable precision trochar with regular medical point needle for easy implantation of small pellets up to 8 mm in diameter as illustrated in Figure 2.3. Since the trochar can only be used for the small pellets up to 8 mm in diameter, the bigger pellets were administered surgically. The animals were anaesthetized under halothane anaesthesia and the pellets were placed in a small subcutaneous pocket above the scapula. The pockets were then surgically sutured closed and the animals were kept warm and allowed to recover from anaesthesia. For the double-pellet implantation groups, i.e. leuprolide or naltrexone (as the first pellet) and testosterone (as the second pellet) group, a 10-day separation period was included between the first and the second administered pellet to let the levels of both testosterone and LH decrease to negligible levels. A jugular bleed was performed to measure baseline levels of LH and A β prior to the implantation of the second pellet, i.e. testosterone pellet. In the absence of LH, the effect of increased doses of exogenous testosterone on A β levels was observed in these groups. All the peripheral pellet insertions were performed by Kevin Taddei and Dr Gardner.

The administration of the pellet in the brain (either LH or placebo) was performed using atropine-diazepam-hypnorm anaesthesia. Briefly, guinea pigs were placed in a stereotaxic head holder and a burr hole drilled in the left and right parietal bones to expose the underlying dura. A pellet were placed in the burr hole on either side of the brain, and held in place on the dura by Gelfoam[®] and dental cement. All animals were

given the analgesic, buprenorphine (0.05 mg/kg BW subcutaneous) immediately post surgery, and kept in a warm place to recover.

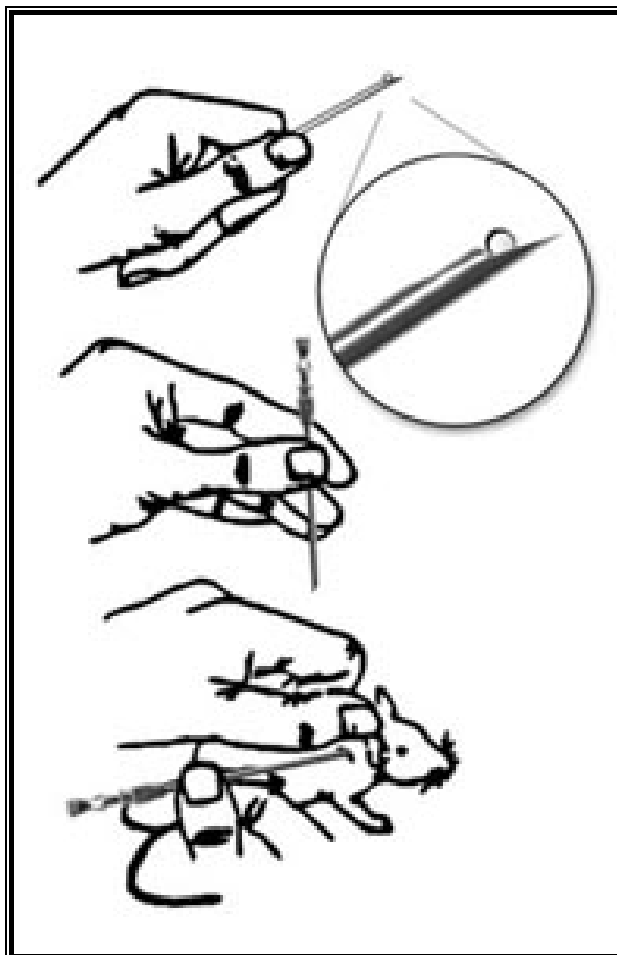


Figure 2.3 : Pellet implantation procedures using a trochar (Innovative Research of America, USA).

2.2.2.2 Animal sacrifice and tissue collection

At the the conclusion of each time point, i.e. day 7, 18 or 36; and day 14 or 28 for the cortical placement group, blood, cerebrospinal fluid (CSF) and the tissues, i.e. brain, liver and kidney were collected. All animals in the control group (baseline) were sacrificed immediately (at time zero) and used to obtain baseline readings of all

parameters for the normal (control) guinea pigs. For each terminal point, the animals were anaesthetised prior to the final bleed. Blood was collected via cardiac puncture for maximal blood collection and CSF was collected by puncture of the cisterna magna. The animals were then euthanized with sodium pentobarbitone immediately following the CSF sample collection. The whole blood and the CSF were then spun at 3500 rpm for 15 minutes at 4°C to collect the plasma and remove the blood cells, respectively. For the tissues collection, all animals were cardiac-perfused with cold saline containing 1000U/ml heparin. The brain was removed and snapped frozen in liquid N₂ for Western immunoblotting and ELISA. All the tissues were then stored in -20°C freezer until used.

2.2.2.3 Preparation and Homogenisation of Brain tissue

Guinea Brains were removed from storage at -20°C and thawed on ice. The brain was then dissected into the following brain regions: frontal cortex, hippocampus and cerebellum. The tissues were then weighed and homogenized using a Teflon-glass homogenizer in 3 ml/g of phosphate buffered saline (PBS) pH 7.4 (mixed powder from Fisher Biotec, Perth, WA) containing a commercially protease inhibitor cocktail (Roche, Basel, Switzerland) prior to the analysis to measure A β and AD related protein levels in the tissue. Some of the total brain homogenates (180 μ l) were spun by airfuge 160,000 x g for 15 minutes to obtain brain extract for α APPs level measurement. Three hundred and ten μ l (310 μ l) of brain homogenates were then processed for ELISA measurements (refer to Section 2.2.4). The remainder was aliquoted and stored at -80°C until use for western immunoblotting.

2.2.3 Western immunoblotting

Western immunoblotting was used to measure semi-quantitative levels of AD related proteins in plasma, CSF and tissue homogenates. This analysis included sample preparation and determination of protein concentration, loading of samples, electrophoresis, and immunoblotting AD related proteins.

2.2.3.1 Preparation and determination of protein concentration

The protein concentration in each region of the brain was estimated using the Micro BCA protein assay kit (Pierce, IL, USA). All homogenates were diluted 1:600, in PBS pH 7.4 and loaded in duplicate into 96-well microtitre plate (100µl/well). Protein extracts used to measure soluble α -APPs were diluted 1/100. Equal amount of standards solutions, made by diluting stock BSA solution (2 mg/ml ddH₂O) to a working dilution of 80 µg/ml (6, 8, 10, 12, 14, 16, 18, 20, 40, 80 µg/ml), and blank (PBS pH 7.4) were also loaded in duplicate in the same manner. One hundred µl of the colorimetric detection mixtures were then added to each well. The plates were then incubated at 60°C for 15 minutes. A BioRad model 3550 plate reader was used to read the absorbance at 595 nm and the protein concentration determined by reference to a standard curve (generated using MCM Plate Manager, BioRad, CA, USA). Each homogenate sample containing 25 µg of total protein was then resuspended in 4X Tris Tricine sample buffer (4g SDS, 2g glycine and 5mg phenol red dissolving in 8.3ml of 1M Tris, pH 6.8) with the addition of double-distilled water [DDW]) containing 0.1M dithiotreitol (DTT) and brought to a final volume of 50 µl. Samples were then stored in -80°C until underwent electrophoresis..

2.2.3.2 Polyacrylamide gel electrophoresis

Tris tricine polyacrylamide gel was used to run all the AD-related proteins. The gel contained a bottom layer of 12% resolving gel (5 cm deep), then an 8% resolving gel (2 cm deep) and a 4% stacking layer to form the wells. For P450scc, the gel contained only the bottom layer of 10% resolving gel (7cm deep) and a 5% stacking layer. The samples and the internal control (IC), which is pooled samples from the rest of the brain, were mixed in sample buffer and together with molecular weight markers (MW) were then vortexed, centrifuged and heated to 95°C for 10 minutes prior to the loading. After the final quick centrifugation, samples, IC, and MW were then loaded and electrophoresed at 80 volt for 30 minutes and then 120 volt for approximately 2.5-3 hours for all proteins or 4.5 hours for P450scc, in cathode (1M Tris, 2M Tricine, 1% SDS) and anode buffer (2M Tris-HCl, pH 8.9) to separate proteins. The gels were removed from the gel caster and the proteins transferred to nitrocellulose membrane by western blotting using

the transfer buffer (25mM Tris, 200mM glycine, 20% methanol), at 250mA, 4°C for overnight or at 350mA, 4°C for 4 hours.

2.2.3.3 Immunoblotting

The membranes were removed from the transfer tank and stained with a Ponceau stain (0.1% Ponceau-S in 5% acetic acid) to determine whether equal protein was loaded and the transfer process was performed well. The picture of these membranes was scanned using GS-800 scanner (BioRad CA, USA). The membranes were then cut into sections, based on specific molecular marker, in order to enable probing for different protein of interest from the same blot. All the sections were then washed in hot PBS several times. All the incubation and washing steps used in the immunoblotting and detection were performed at room temperature with gentle rocking .

Non-specific binding sites of protein were blocked using blocking buffer (TBS containing 5% skim milk) for 1 hour. The blocking buffer was drained, then TBST pH 7.4 (TBS containing 0.05% (v/v) Tween-20) containing 0.05% skim milk were used to dilute the primary antibodies. All the primary antibodies used to detect proteins of interest along with the dilutions are listed in Table 2.5.

Table 2.4 Primary antibodies used for western blotting (*p* denotes polyclonal, *m* denotes monoclonal)

Antibody	Protein	Dilution used	Source of antibody
<i>m</i>C1/6.1 by Dr. Paul Matthews (Nathan Kline Institute, NY, USA)	APP	1:5,000	Mouse
	C100	1:5,000	
<i>m</i>WO2 by Professor Colin Masters (University of Melbourne, Vic, Australia)	α APPs	1:2,000	Mouse
<i>m</i>GFAP (Sigma chemical)	GFAP	1:10,000	Mouse

Co., Missouri, USA)			
papoE (Milipore)	apoE	1:10,000	Goat
pP450scc (Milipore)	P450scc	1:1000	Rabbit
mβ-actin (Sapphire Bioscience Pty Ltd, Redfern, NSW, Australia).	β-actin	1:20,000	Mouse

The membranes were incubated in the primary antibody for 2 hours and then washed 3 times for 10 minutes with TBST pH 7.4. The secondary antibody linked to horseradish peroxidises (HRP) was then diluted in TBST pH 7.4 containing 0.05% skim milk and used to incubate the membranes for 1 hour. All the secondary antibodies used in the western immunoblotting are listed in Table 2.6

Table 2.5 Secondary antibodies used for western blotting (all antibodies are linked to HRP)

Antibody	Protein	Dilution used
Sheep anti-mouse antibody	APP, C100, GFAP, αAPPs	1:5,000
	β-actin	1:10,000
Rabbit anti-goat antibody	apoE	1:10,000
Donkey anti-rabbit antibody	P450scc	1:5,000

The membranes were washed with TBST pH 7.4 (3x10 minutes) and rinsed with TBS. Finally, the membranes were incubated in Enhanced Chemiluminescence reagent (ECL, Amersham Biosciences, UK) for 2 minutes with a fast rocking motion and then blotted

and exposed to ECL hyperfilm (Amersham Biosciences, UK) with different exposure time in order to get the desired signal intensity. All the film were developed in the dark room and scanned for semi-quantitative analysis using Quantity One[®] 1-D Analysis Software (BioRad, CA, USA).

2.2.4 Brain ELISA sample preparation

Brain homogenate (310 μ l) in PBS containing protease inhibitor was resuspended in 710 μ l of brain ELISA buffer (250mM sucrose, 20mM Tris-HCl, 1mM EGTA [Ethylene glycol-bis (2-amino-ethylether)-N,N,N',N'-tetra-acetic acid], 1mM EDTA [Ethylenediaminetetra-acetic acid], pH 7.4,. A volume of 1.1ml cold 100mM NaCl containing 0.4% diethylamine (DEA) was then added to the brain homogenates and mixed using a Teflon-glass homogenizer. The homogenate was then centrifuged at 1000.000G, 4°C for 40 minutes for all the treatment group except for groups with LH and placebo implantation in the brain which were spun at 100,000G, 4°C for 1 hour. A 1.7ml aliquot of supernatant was mixed with 170 μ l of 0.5M Tris-HCl, pH 6.8, and aliquoted into 4 tubes (~ 440 μ l/tube) and then stored in -80°C until use. Each aliquot were used to measure A β ₄₀ and A β ₄₂ levels by ELISA, as well as the CSF samples.

2.2.5 Enzyme-Linked ImmunoSorbent Assay (ELISA)

The ELISA was used to measure A β ₄₀ and A β ₄₂ levels in plasma, CSF (from human and guinea pigs), and brain homogenates (from guinea pigs) As levels of A β in the CSF are very high (above the highest standards of ELISA), the CSF was diluted in PBS (1:10) before the analysis. All the frozen samples were thawed on ice prior to the analysis.

The ELISA protocol was as follows: Nunc 96 well plates were coated with 100 μ l/well of coating primary (6E10) antibody solution (25 μ l of primary antibody in 10ml of CO₃/HCO₃ solution [0.26g Na₂CO₃, 0.21g NaHCO₃, pH 9.6 with final volume of 50 ml was met with addition of DDW]). As guinea pig A β is identical to human A β (Beck, 1997), the same primary antibody 6E10 was used for both human and guinea pig samples. The plates were sealed and incubated at 4°C overnight. Plates were then

washed 3 times with PBST pH 7.4 using the plate washer (BioRad, CA, USA) and blocked with blocking buffer 200 μ l/well (0.25g of BSA (Bovine Serum Albumin) diluted in 25ml PBST) at room temperature for 1 hour, followed by 3 washes in PBST. Samples and standards were then loaded into the wells of the plates (100 μ l/well). The plates were sealed and incubated at room temperature for 2 hours and then at 4°C overnight. The next day, the plates were washed 3 times with PBST, then 100 μ l/well of detection antibody solution (10 μ l of secondary antibody [M40 for A β ₄₀ or R301 for A β ₄₂] diluted in blocking buffer) was added. The plates were sealed and incubated at room temperature for 1.5 hours, followed by 3 washes with PBST. The neutravidin-HRP solution (100 μ l/well) was then added into the wells, and the plates were incubated at room temperature for 1 hour. The plates were then washed again with PBST prior to adding TMB/KGP reagent (100 μ l/well). The plates were shielded from the light to allow the reaction of TMB/KGP to take place until the wells of the last 2-3 highest standard were distinguishable (usually took between 10-15 minutes). A 1M H₃PO₄ solution (100 μ l/well) was used to stop the TMB/KGP reaction. The plates were then read in a BioRad microplate reader at 450nm to provide of a raw OD reading of A β levels. The unit of the A β calculation depends on the amount of A β detection in each samples measured (in nanomolar for the brain homogenates samples and picogram or nanogram for CSF and plasma samples). The A β results obtained from calculation of A β standard concentrations by comparing the sample absorbance with the absorbance of known concentrations of A β ₄₀ or A β ₄₂ standards assayed identically on the same plate. Using the wet weight of the brain in the original homogenate, the final values of A β in brain were expressed as nanomoles / gram wet weight or picogram/ ml and nanogram / ml.

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Chapter 3

Reduction of CSF and cerebral A β levels by testosterone in castrated guinea pigs

3.1 Introduction

Studies of estrogen levels and the incidence of AD in women are numerous, and have shown that hormone therapy around the time of menopause (but not in much older women) may delay the onset of AD (Brinton *et al.*, 2004) *In vitro* studies supporting this effect have shown that estrogen can reduce the production of A β in culture, and promote the secretion of α -APPs, another proteolytic product of APP known to have neurotrophic properties (Brinton *et al.*, 2004) As *in vitro* studies have shown that testosterone can also reduce production of A β , promote α -APPs secretion and promote neuronal viability (Goodenough, 2000 ; Gouras, 2000 ; Pike, 2006), possible links between testosterone and AD are now being investigated.

Levels of testosterone on average decline in men by approximately 1% every year from the 3rd - 4th decade of life, and the gradual decline in androgen levels that occurs, combined with other factors that increase resistance to androgen action, such as rising sex hormone binding globulin (SHBG) levels with age, causes androgen deficiency symptoms often referred to as andropause (Haren, 2002 ; Pike, 2006). It has been suggested that the above-mentioned effects of testosterone do not occur via androgen receptors (AR), but occur following aromatisation to estradiol which then binds to estrogen receptors (ER). The use of non-aromatisable dihydroxytestosterone (DHT) in some of these androgen experiments, and aromatase inhibiting drugs in others, has confirmed that some (but not all) of the *in vivo* and *in vitro* effects of testosterone can be attributed to AR pathways (Pike, 2006).

An association between low testosterone levels and AD is supported by recent findings that testosterone levels are inversely correlated with plasma A β levels (Gillet, 2003). Low free testosterone has now been found to be an independent predictor of AD, and elderly AD cases also appear to have raised levels of gonadotropins (Hogervorst, 2003 ; Hogervorst, 2004) Another study has found that brain testosterone levels in subjects with mild neuropathology are similar to levels in subjects with more advanced AD, yet significantly lower than age-matched controls (Rosario, 2004). In a large longitudinal study, it was found that low free testosterone levels could even be detected 5-10 years prior to diagnosis of AD (Moffat, 2004) Not all studies agree with the above findings (Geerlings, 2006) nevertheless, chemical castration therapy in men with prostate cancer

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results in increased plasma A β levels, providing alternative *in vivo* proof that testosterone can influence A β levels (Gandy, 2001 ; Almeida, 2004). *In vivo* studies also contradict some of the *in vitro* findings, e.g. whereas many cell culture studies have suggested testosterone effects occur via ER following aromatisation to estrogen, *in vivo* studies of castrated animals show the non-aromatisable androgen dihydrotestosterone (DHT), and not estrogen, influenced A β levels (Ramsden, 2003). The aim of this chapter is to investigate further the role of testosterone in A β metabolism *in vivo*, using the guinea pig (*Cavia porcellus*) as an animal model. In addition to a complete sequence identity to the human A β protein (Beck, 1997), this non-transgenic rodent model offers the advantage of yielding greater amounts of brain tissue and CSF samples than can be collected in mice and rats. Further, guinea pigs have a similar cholesterol metabolism to humans (Luz Fernandez, 2001 ; Zern, 2003 ; Sharman, 2008), which is important in synthesis of sex steroids and in AD pathogenesis. As a non-transgenic animal model, the guinea pig also provides the opportunity to investigate any subtle effects of hormones or gonadotropins that may be masked by over-expression of APP in transgenic murine models. A number of other studies have utilized the guinea pig as a model for investigating AD related proteins (Beck, 1997 ; Fassbender, 2001; Beck, 2003) and evaluating the efficacy of inhibitors and modulators of A β production (Netzer, 2003 ; Lanz, 2005). Guinea pigs have also been used to evaluate the effects of oestrogen on brain A β levels (Petanceska, 2000), where depletion promoted A β accumulation, which was alleviated with supplementation of this hormone. In this chapter, I describe the effects of testosterone depletion and supplementation on CNS A β levels. Previous findings have reported correlations between testosterone and peripheral levels of A β (Gandy, 2001 ; Gillet, 2003 ; Almeida, 2004) thus I also assessed the effects of testosterone reduction and supplementation on plasma A β and investigated whether there is a relationship between changes in plasma testosterone levels and altered CSF and cerebral A β levels.

3.2 Aims

The aims of the study were:

- To determine if CSF and plasma A β levels are altered in the presence of reduced testosterone levels in gonadectomised (GDX) guinea pigs.

- To determine if testosterone supplemented to gonadectomised (GDX) guinea pigs restored CSF and plasma levels to that seen in non-gonadectomised (non-GDX) guinea pigs.
- To determine if a reduction in testosterone or supplementation of this hormone alters brain levels of A β or APP and its metabolites.

3.3 Methods

Animals

Seventy eight adult male guinea pigs, body weight (BW) 500 gram (\pm 6-8 weeks old), were used. During the experiments, animals were kept in a 1:1 light and dark cycle room with free access to a soy-free diet (Glen Forrest Stockfeeders, Perth, WA) and water. A summary of the animal groups are described in Figure 3.1.

Twenty three animals were left intact (non-GDX) and 55 animals were then castrated (GDX) under halothane anaesthesia. One week following castration, 40-day slow release pellets (Innovative Research of America, Florida, USA) were implanted in the periphery (subcutaneous insertion into the dorsal neck fat pad of guinea pig), as described in section 2.2.2.1. The pellets contains placebo (n=12), low testosterone (2.5 mg/kg Body Weight (BW)/day, n=12) or high testosterone (20 mg/kg BW/day, n=12), The remaining GDX animals were left without any treatment (n=19).

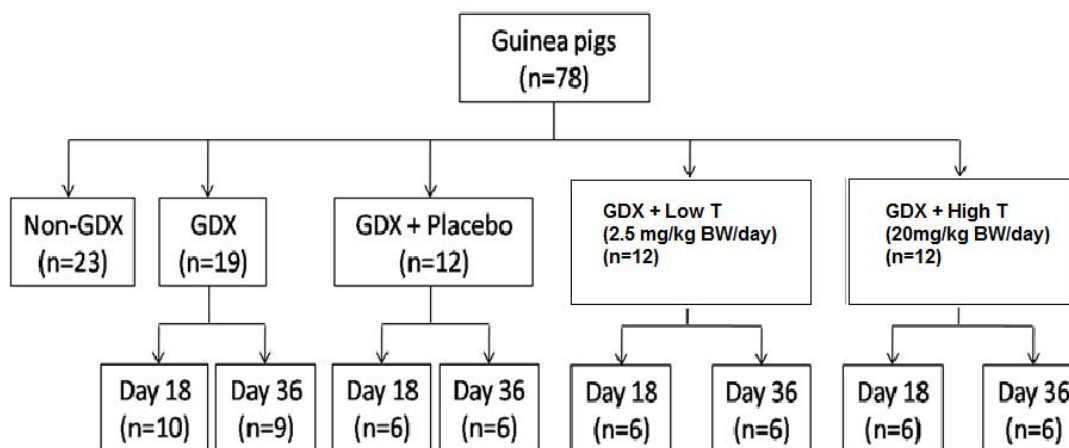


Figure 3.1: A Summary of animal groups used in the study.

CSF, Plasma and Tissue Collection

The CSF, plasma and tissue collection was performed as described in section 2.2.2.2 and 2.2.2.3. Briefly, at each point of sacrifice, (i.e. 0, 18, 27, 36 days), animals were anaesthetised with halothane and blood was collected via cardiac puncture. CSF was collected from the cisterna magna. Processing of whole blood (to collect plasma) and CSF, prior to analysis of A β 40 levels is described in section 2.2.2.2. To collect brain tissue, all animals were cardiac-perfused with heparinized-cold phosphate buffer saline (PBS) and the brain removed. The brains were dissected into regions, such as frontal cortex, hippocampus and cerebellum, and homogenized in PBS containing protease inhibitor. Brain homogenates were used for protein analysis of the APP metabolites protein, Full length (FL) APP, secreted α -APP (s- α APP) and APP C-terminal fragment (APP-CTF) were assessed by western immunoblotting as described in section 2.2.3.3, A β 40 levels were assessed by ELISA (as described in section 2.2.5).

Measurement of serum testosterone, plasma and CSF A β 40 levels.

Serum testosterone was measured by Path Centre (Perth, Australia) and plasma and CSF A β 40 levels were measured by sandwich ELISA using 6E10 as capture antibody (anti-human A β , Signet Laboratories, USA) and R208 as a detection antibody (kindly provided by Dr. Pankaj Mehta, NYS Institute for Basic Research, New York, USA) (see chapter 2.2.5).

Statistics

All data that was normally distributed were analyzed using ANOVA at the 0.05 significance level. The levels of testosterone, CSF A β 40 levels, FL-APP and its C-terminal fragment were found to be not normally distributed and thus were analyzed using non parametric Kruskal Wallis analysis of variance.

3.4 Result

3.4.1 Distinct effects of testosterone reduction or supplementation on CSF and plasma A β 40 levels.

Guinea pigs were either left intact (non-GDX), GDX only or GDX and supplemented with low (2.5 mg/Kg BW) or high (20 mg/kg BW/day) concentrations of testosterone. The animals were initially sacrificed 18 and 36 days after treatment and serum, CSF and plasma were collected to measure testosterone and A β 40 levels, respectively. These results are shown in Figures 3.2-3.4. Please note that groups of GDX guinea pigs administered placebo implant were also included. However, all animals in this group did not show a value for plasma A β levels, despite obtaining values for all other groups. The assay was performed three times without obtaining a value for samples in the GDX +placebo group. However, serum testosterone and CSF A β levels were obtained for the GDX+ placebo and shown to have similar levels to the GDX only [Testosterone (Mean \pm SEM) nmol/L: GDX = 2.74 ± 0.31 , GDX+placebo = 2.76 ± 0.17 ; CSF A β 40 (Mean \pm SEM) ng/ml ; GDX = 2.65 ± 0.38 , GDX+placebo A β 40 = 2.54 ± 0.33] Since the aim was to determine the effects of testosterone on plasma A β and investigate if there is a relationship with any changes observed in the CSF and brain, results from the GDX only group were included to represent a reduction in testosterone and comparisons were made with this group.

To confirm that GDX resulted in a reduction in testosterone concentration and that supplementation resulted in an increase in concentration, serum levels were measured. Compared to non-GDX controls, significant reductions in testosterone levels ($p < 0.05$) were observed following 18 or 36 days GDX (Figure 3.2). This decline was associated with a significant increase in CSF A β 40 levels at days 18 and 36 following GDX (see Figure 3.3). Compared to non-GDX animals, plasma A β levels were similar following 18 days GDX, but were significantly increased after 36 days GDX (Figure 3.4).

Administering the low dose testosterone pellets for 18 days caused a rise in serum testosterone levels to that seen in non-GDX animals (Figure 3.2). This was accompanied by a decrease in plasma A β 40 to levels seen in non-GDX animals, yet CSF A β 40 levels were almost as high as in the untreated GDX animals (Figure 3.3). By day 36, serum testosterone (Figure 3.2) and plasma A β 40 levels (Figure 3.4) were back to levels seen in the untreated GDX animals. In contrast, CSF A β 40 levels were lower than those seen in untreated GDX animals, closer to A β 40 levels found in non-GDX control animals (see Figure 3.4).

The administration of pellets containing high testosterone resulted initially in supra-physiological serum testosterone levels at day 18, reducing to levels found in non-GDX controls by day 36 (see Figure 3.2). An additional group at which GDX guinea pigs were administered testosterone for 27 days was included to determine if this reduction was time dependent. The results show that a gradual reduction in testosterone following 27 and 36 days exposure to the testosterone implant. Despite these initial supra-physiological levels of testosterone, A β 40 levels in CSF at day 18 and day 27 were not significantly reduced compared to untreated GDX animals. A significant reduction in CSF A β 40 levels was only detected on day 36 ($p < 0.05$) (Figure 3.3). On day 18, plasma A β 40 levels in the high testosterone group were similar to levels found in the plasma of untreated GDX animals (on day 18), despite the supra-physiological levels of circulating testosterone. At day 27 and 36 of treatment, plasma A β 40 levels increased significantly above the GDX or Non-GDX guinea pig test group A β 40 levels (see Figure 3.4).

Overall reducing testosterone, via castration significantly increased CSF and plasma A β 40 levels in experimental guinea pigs. Supplementation of testosterone had distinct effects on plasma and CSF A β 40, where levels were reduced in CSF but increased in plasma. I next determined if brain A β 40 levels were altered as a response to changes in testosterone levels and if this was associated with alterations in APP processing.

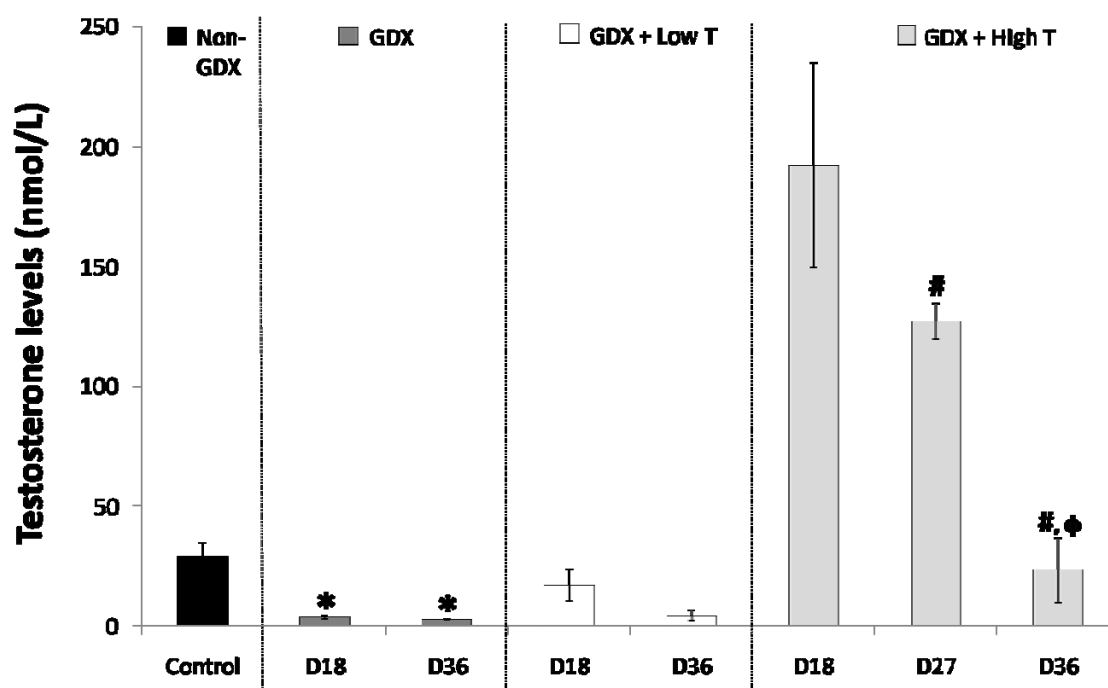


Figure 3.2: Serum testosterone levels in non-GDX, GDX and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment. An additional group at which GDX guinea pigs were administered a high dose of testosterone for 27 days was included. All data are shown as mean \pm SEM. T=testosterone and D=day of treatment. * $p < 0.05$, testosterone levels significantly reduced compared to non-GDX animals. # $p < 0.05$, testosterone levels significantly increased from corresponding untreated GDX animals. Φ $p < 0.05$, testosterone levels significantly reduced from GDX animals supplemented with testosterone for 27 days.

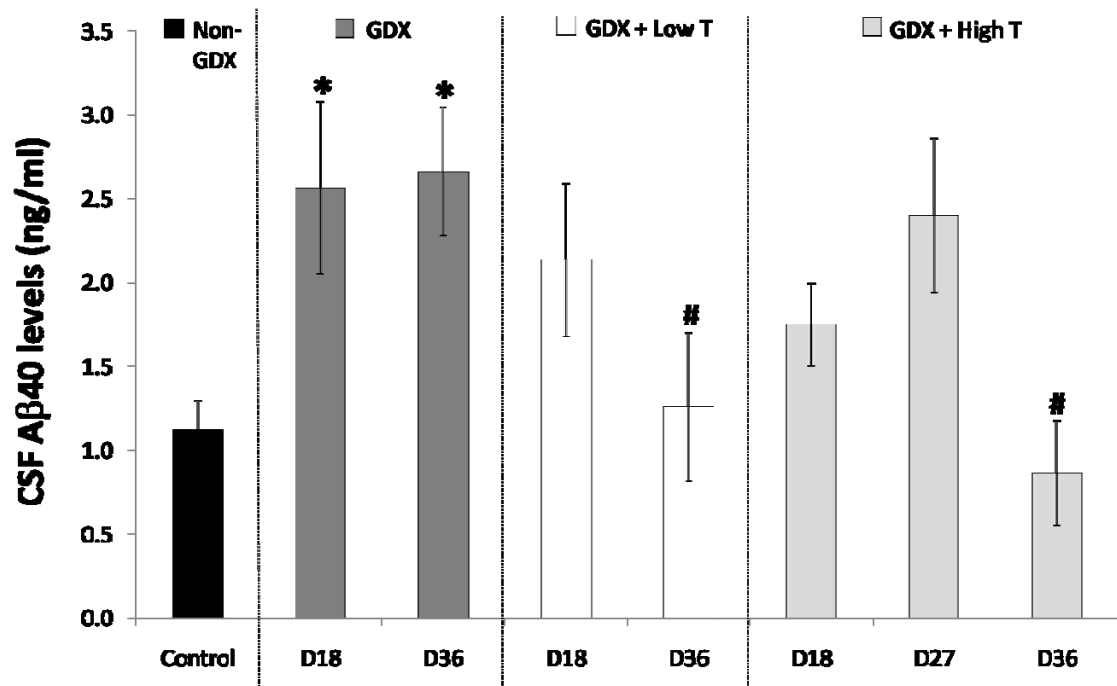


Figure 3.3: CSF Aβ40 levels in non-GDX, GDX and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment. An additional group of GDX guinea pigs which were administered a high dose of testosterone for 27 days was included. T=testosterone and D=day of treatment. * p<0.05, CSF Aβ40 levels are significantly increased compared to non-GDX animals. # p<0.05, CSF Aβ40 levels are significantly lower compared to untreated GDX animals, at day 36.

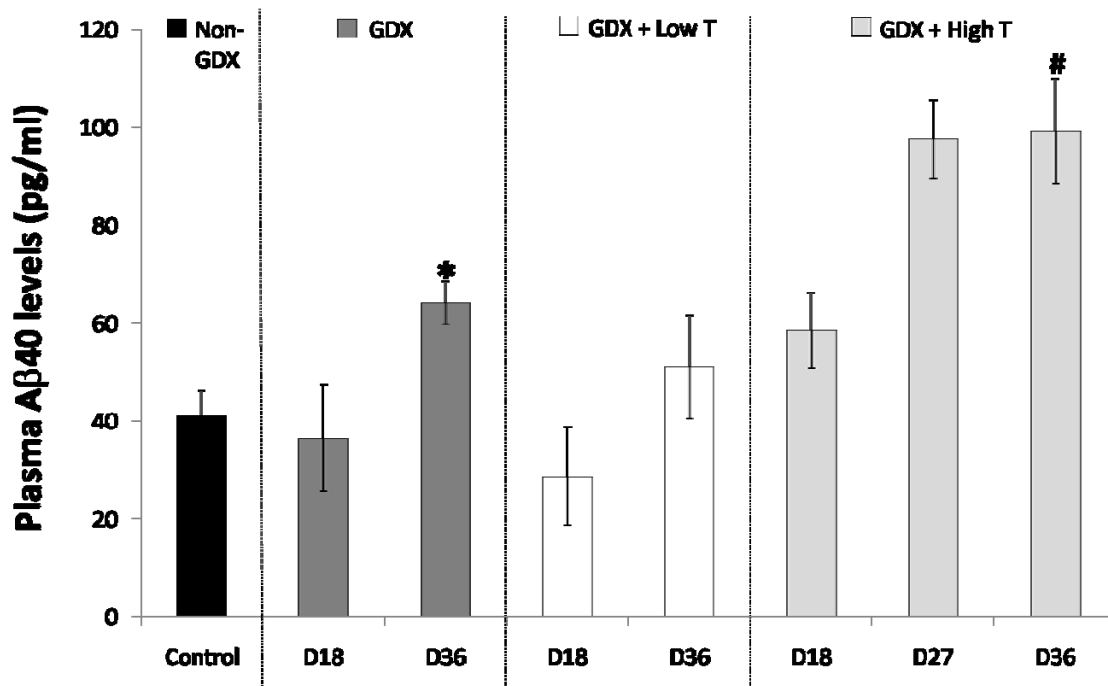


Figure 3.4: Plasma Aβ40 levels in non-GDX, GDX and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment. An additional group at which GDX guinea pigs were administered a high dose of testosterone for 27 days was included. T=testosterone and D=day of treatment. * p<0.05, plasma Aβ40 levels are increased compared to non-GDX animals at day 36. # plasma Aβ40 levels are significantly increased compared to GDX at day 36.

3.4.2 Effects of testosterone reduction or supplementation on brain Aβ levels and APP processing.

To determine if testosterone altered brain Aβ40 levels, the frontal cortex, hippocampus and cerebellum from each experimental group underwent analysis. The results are shown in Figure 3.5. Compared to non-GDX control animals, Aβ40 levels in the frontal cortex and cerebellum were not significantly altered 18 days after GDX (Figure 3.5 A and C). However, a significant reduction was observed following administering a high

dose of testosterone for 18 days. This reduction was not observed following 36 days of testosterone treatment. It was noted, however, that compared to non-GDX, GDX led to a reduction of A β 40 in the frontal cortex and cerebellum. This may account for the lack of effect of testosterone at day 36, as levels were already reduced in GDX animals. A significant increase was observed in the hippocampus of GDX animals, at day 18 which was significantly reduced below the levels shown in non-GDX animals following administering testosterone at highest dose used in this study (20mg/kg BW/day) (Figure 3.5 B). Although the effect of GDX was lost at day 36, supplementation of testosterone resulted in a reduction in A β 40 levels at this time point.

Testosterone has been shown to impact on A β production *in vitro* and *in vivo*, through altering APP processing (Gouras, 2000³ ; Pike, 2006⁴) . To determine if APP metabolism is altered in the GDX guinea pig in the presence or absence of testosterone, levels of APP and its metabolites (APP-C-terminal fragments (APP-CTF) and secreted APP (α -APPs) were assessed. (Figure 3.6-3.8). Levels of FL-APP (Figure 3.6) and APP-CTF (Figure 3.7) were not significantly altered as a result of castration nor testosterone supplementation. Analysis of sAPP α was performed using tissue from the frontal cortex. This region provides sufficient material for ultracentrifugation to obtain supernatant-free of cell debris (as described in section 2.2.2.3). Following A β analysis via ELISA and western immunoblotting analysis of APP, the frontal cortex was the only region to have sufficient amount of tissue remaining required to perform this analysis. The sAPP α protein was detected via western immunoblotting and underwent quantitative analysis. The findings (Figure 3.8) showed that compared to non-GDX animals, a significant reduction in α -APPs levels was observed in the frontal cortex of GDX animals. The supplementation of testosterone, either low or high levels, restored the levels of α -APPs to that seen in non-GDX animals (see Figure 3.8).

Overall the results show that a reduction in testosterone as a result of GDX is associated with a reduction in α -APPs . The addition of testosterone following GDX restored α -APPs levels to that seen in the non-GDX guinea pigs. Although most prominent in the hippocampus, testosterone also reduced brain A β 40 levels. Together, these results

indicate that testosterone may modulate brain APP processing through the non-amyloidogenic pathway. Parallels were also observed between brain, CSF and plasma A β 40 for some of the experimental treatments. In particular, treatment with the highest dose of testosterone led to reductions in brain and CSF levels but increased plasma A β 40.

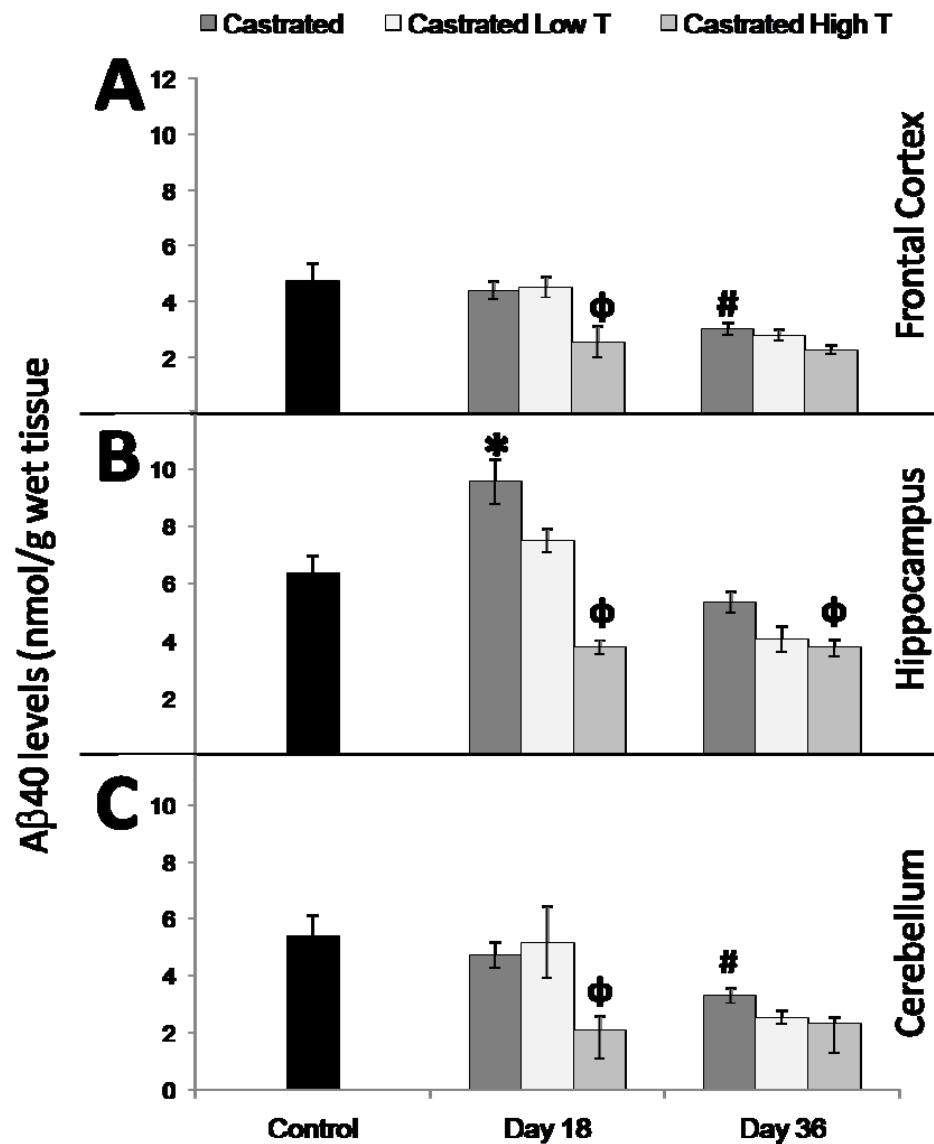


Figure 3.5: A β 40 levels in frontal cortex, hippocampus and cerebellum from non-GDX, GDX only and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment. Homogenates from the (A) Frontal cortex, (B) Hippocampus and (C) Cerebellum underwent ELISA analysis for A β 40. Data is represented as mean A β 40 levels (nmol/g wet tissue weight) \pm SEM. T= administered testosterone pellet. *, p<0.05, A β 40 levels increased compared to non-GDX animals. #, p<0.05, A β 40 levels decreased compared to non-GDX animals. ^Φ, p<0.05, A β 40 levels decreased from GDX animals.

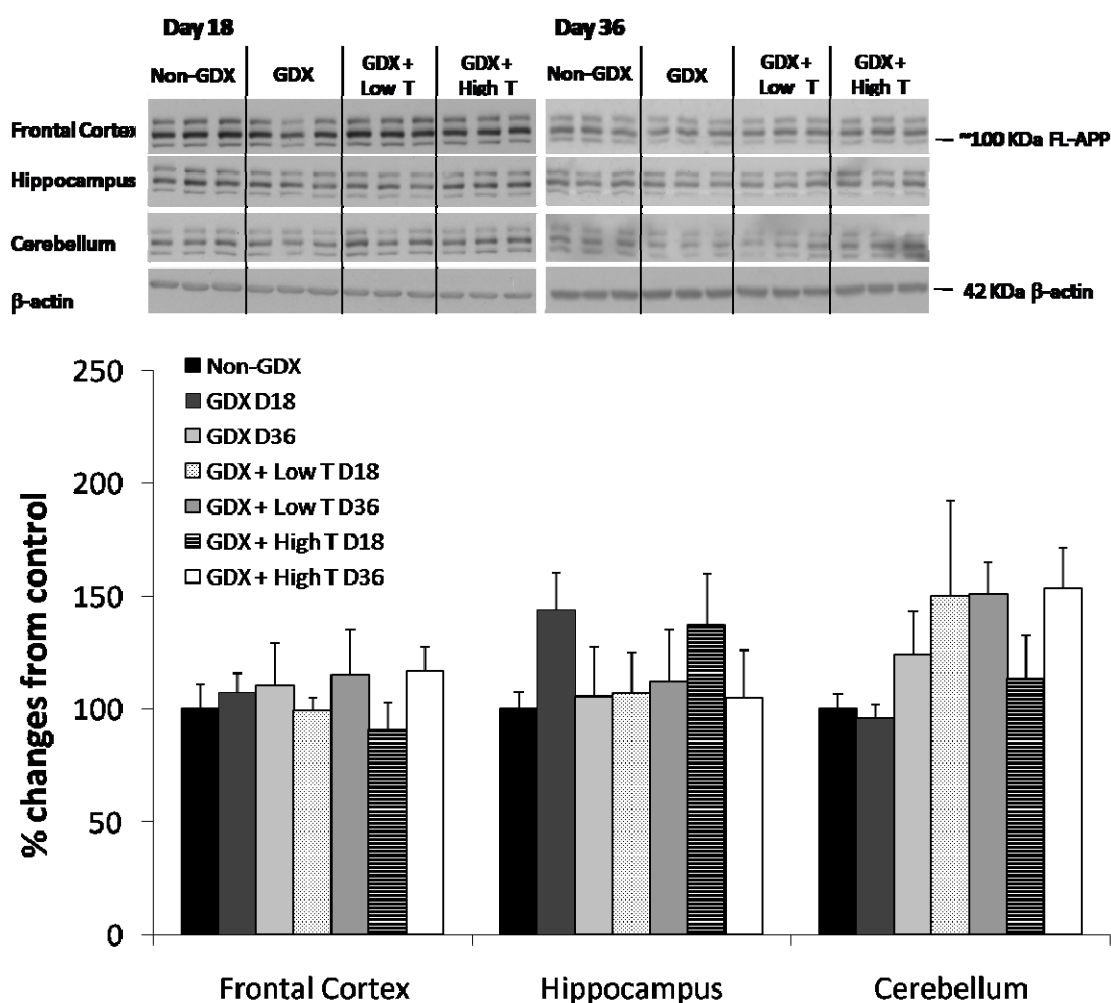


Figure 3.6: Full length (FL) APP levels in frontal cortex, hippocampus and cerebellum from non-GDX, GDX only and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment. (A) Homogenates from the frontal cortex, hippocampus and cerebellum underwent western immunoblotting for full length (FL-APP) using antibody C1/6.1 or β-actin. Three samples from each group are represented on the immunoblots. (B) Quantitative analysis of all samples revealed no overall significant changes in FL-APP levels in any of the brain regions analysed. Data is represented as percentage of non-GDX control and are shown as mean ± SEM.

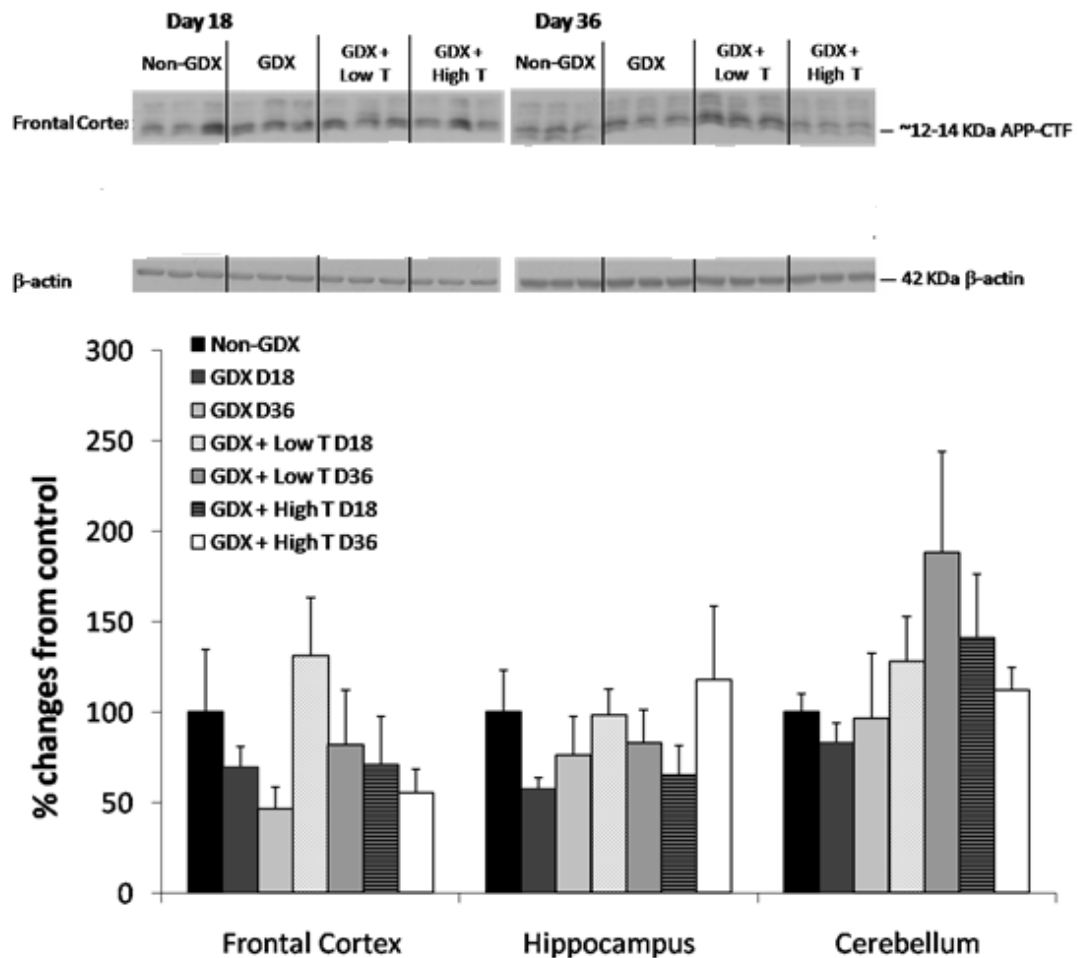


Figure 3.7: APP-CTF levels in frontal cortex, hippocampus and cerebellum from non-GDX, GDX only and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment (A) Homogenates from the frontal cortex, hippocampus and cerebellum underwent western immunoblotting for APP-CTF using antibody C1/6.1 or β-actin. Three samples from each group are represented on the immunoblots. (B) Quantitative analysis of all samples revealed no overall significant changes in APP-CTF levels in any of the brain regions analysed. Data is represented as percentage of non-GDX and are shown as mean ± SEM.

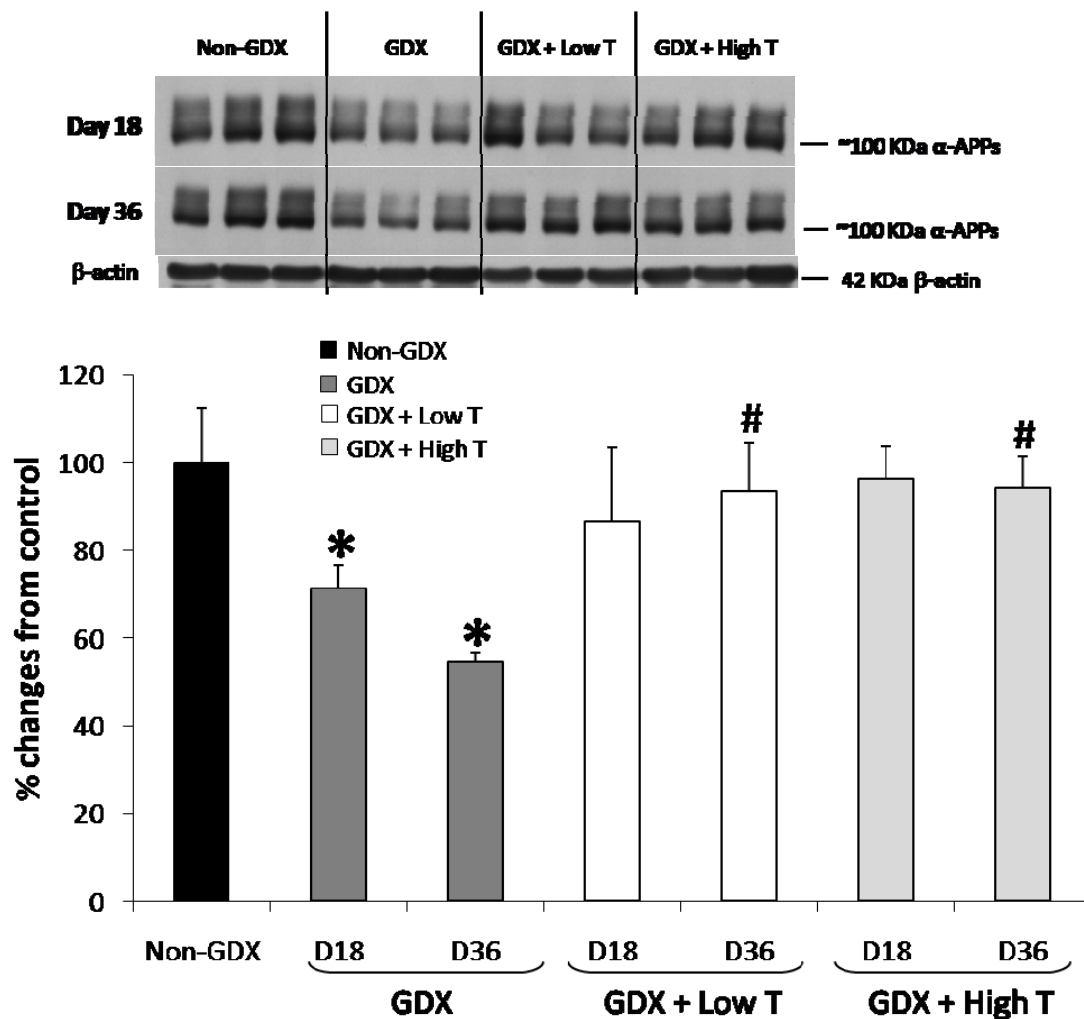


Figure 3.8: Levels of α -APPs levels in the frontal cortex, from non-GDX, GDX only and GDX guinea pigs administered testosterone. Non-GDX animals were sacrificed at baseline (day 0) GDX only or GDX guinea pigs supplemented with low (2.5 mg/kg BW/day) or high (20 mg/kg BW/day) doses of testosterone were sacrificed at 18 and 36 days following treatment (A) Homogenates from the frontal cortex were western immunoblotted for α -APPs using antibody WO2. Immunoblots exhibit 3 representative samples from each group. (B) Quantitative analysis of all samples revealed a significant reduction in levels of α -APPs in GDX animals, which were restored to those observed for the non-GDX animals following testosterone treatment. Data is represented as percentage of non-GDX and are shown as mean \pm SEM.

α -sAPP levels are significantly reduced (*, $p < 0.05$,) from those observed in the intact control. α -APPs levels are significantly increased in the testosterone treated animals compared to the castrated GDX animals. (# $p < 0.05$)

3.5 Discussion

Replacement of testosterone or its metabolite, DHT to mice has been shown to improve learning and memory and reduce cerebral amyloid accumulation (Ramsden, 2003; Rosario, 2006 ; Cherrier, 2007). This chapter describes the use of a non-transgenic animal model to further investigate the benefits of testosterone to reduce A β accumulation. The guinea pig unlike mice, shares A β sequence identity and more closely mimics cholesterol metabolism as well as hormonal changes seen in humans and has the added advantage of having more available tissue (ie brain, CSF and plasma) for experimentation. This has enabled the correlation between testosterone and CSF in particular to be determined A β thereby extending the the correlation between testosterone and plasma A β studies performed in mice.

To model testosterone depletion, guinea pigs were castrated and left for 18 or 36 days prior to analysis of serum testosterone, and A β levels in the periphery and CNS. As expected, serum testosterone levels significantly declined following castration, and this was associated with increased levels of CSF and plasma A β 40. Other *in vivo* studies have also found that lowering testosterone levels either by orchidectomy or anti-androgen therapy results in increased plasma A β levels (Gandy, 2001 ; Almeida, 2004) or brain A β levels (Ramsden, 2003 ; Rosario, 2006). Additionally, the lower testosterone levels resulting from castration cause a loss of negative feedback of testosterone on the luteinizing hormone (LH), a gonadotropin, leading to elevated LH levels, and this has also previously been shown *in vitro* to favour the amyloidogenic pathway, increasing A β production (Bowen, 2004) The inclusion of plasma LH values in this study would have provided greater insight into whether changes in the HPG axis contributed to the effects observed. However, this was not possible as unfortunately there are no standard assays to measure guinea pig LH levels. The β -unit of guinea pig LH has little or no homology with human or rodent LH, thus appropriate, reliable immunoassays for measuring guinea pig LH are not available. However, since guinea pig LH has the highest homology with sheep LH, we attempted to measure the samples

using a radio-immunoassay for sheep LH but serum values using this assay were undetectable. Considering that a change in the hormone levels causes a dysregulation of the HPG axis, it is conceivable that testosterone may alter A β levels indirectly through modulating LH levels.

Androgen receptor (AR) levels in the brain will also have been affected by gonadectomy and subsequent androgen treatment. Testosterone treatment increases levels of AR phosphorylation, which has been shown to increase AR transcriptional activity, whereas in gonadectomised mice, testosterone treatment causes a drop in AR synthesis, possibly due to AR promotor methylation (for a review see Fuller, 2007). The testosterone treatment in the current experiments will have induced changes to many of the above-mentioned components of the hypothalamic-pituitary-gonadal axis, the complex interaction of which will have produced the detected changes in A β levels.

Many *in vitro* testosterone studies have found that decreasing production of A β via the amyloidogenic pathway is coupled to an increase in the secretion of α -APPs, produced via the alternative non-amyloidogenic pathway (Goodenough, 2000 ; Gouras, 2000 ; Pike, 2006), and increased α -APPs levels are thought to be neuroprotective. However not all *in vitro* studies have found these pathways to be coupled (Fuller, 1995), and the negative correlation between A β levels and α -APPs levels seen in *in vitro* androgen or estrogen studies has not been found in *in vivo* studies (Petanceska, 2000 ; Ramsden, 2003 ; Pike, 2006). For example, gonadectomised mice have increased brain levels of A β when compared to control mice, and treatment of gonadectomised animals with the non-aromatisable DHT reduces brain A β levels down to levels below that found in intact animals, yet with no effect on α -APPs (Ramsden, 2003). In this chapter, I have shown that testosterone reduced CSF and cerebral A β levels and restored the reduction in α -APPs resulting from gonadectomy, indicating that testosterone may modulate APP processing through the non-amyloidogenic pathway.

One explanation in the difference between my results and those reported by Ramsden and colleagues, 2003 may be due to the type of androgen used. The aromatisation of testosterone to estrogen is thought to be involved in the apparent testosterone induced secretion of APP, as inhibition of aromatase in the presence of testosterone results in a reduction in α -APPs (Goodenough, 2000). Furthermore, estrogen itself has been shown to induce α -APPs secretion (Bi, 2000 ; Manthey, 2001). Thus it is plausible that testosterone could be altering APP metabolism through estrogen dependent pathways. However, DHT which does not undergo aromatisation to estrogen may have effects on APP metabolism and A β production which are independent of estrogen or the enzymatic processing of APP as reported in other studies (Ramsden, 2003 ; Rosario, 2006). Whether DHT or testosterone has independent effects will need to be clarified in studies that directly compare the effects of these androgens on APP metabolism.

The findings in this chapter show that low dose testosterone decreased A β 40 levels, resulting in a negative correlation between plasma A β 40, plasma testosterone and α -APPs levels. This is similar to results found by others who employed either *in vitro*, animal or human studies (Gouras, 2000 ; Gillet, 2003 ; Ramsden, 2003). However, CSF A β 40 levels did not mirror the changes seen in blood plasma. If anything, changes in CSF A β 40 levels appeared to lag behind changes in plasma testosterone and plasma A β 40 levels by a period of 1-2 weeks. This is conceivable if changes in other components of the hypothalamic-pituitary-gonadal axis control A β 40 levels in the CSF (for example, LH levels), as opposed to a more direct action following androgens binding to androgen receptors. Gonadectomy followed by fluctuating testosterone levels induced by the experimental treatment would cause ongoing re-equilibration in the hypothalamic-pituitary-gonadal system, until testosterone returned to a constant low level. Alternatively, or in addition, CSF A β levels are in general much higher than circulating levels of A β , leading us to speculate that our results may reflect slow clearance of A β from the CSF to the bloodstream. The clearance process of A β from brain to bloodstream involves various stages, including and not limited to (1) diffusion between the interstitial fluid (ISF) and capillaries, (2) the continuous slow removal of A β via the ISF-CSF bulk flow into the bloodstream, (3) mediated clearance processes

by carrier proteins such as apolipoprotein E, apolipoprotein J, α 2-macroglobulin, and (4) transport of A β across the brain-blood barrier regulated by the receptor for advanced glycation end products and the low density lipoprotein receptor protein-1 (LRP) (Silverberg, 2003 ; Deane, 2004) [Silverberg, 2003; Deane, 2004]. Once in the bloodstream, A β has a short half-life and is believed to be rapidly degraded by the liver and kidneys (Hone, 2003).

Feedback mechanisms and components of the hypothalamic-pituitary-gonadal axis following castration in young male guinea pigs, e.g. AR, LH and follicle stimulating hormone (FSH) levels and testosterone negative feedback effects, are unlikely to be identical to those of aging human males with low, but not absent, levels of androgens. However, this study provides additional evidence of a role for testosterone in A β metabolism, and supports the concept that androgen therapy regulates brain A β levels and hence reduces the risk of AD in men. The high plasma A β 40 levels in the animals treated with the higher dose of testosterone are of particular interest. Such high levels of testosterone would be expected to induce aromatisation to estrogen, which has been shown to reduce A β levels in female rodent brains, and is thought to improve memory in elderly men (Ramsden, 2003 ; Cherrier, 2007). However, this result does not support the concept that supra-physiological levels of testosterone will further reduce A β levels. The significant lowering of CSF A β 40 levels only occurred by day 36, by which stage circulating testosterone levels were either within the normal range or below normal levels. The results are in agreement with results by Ramsden *et al.*, 2003. who also found that changes in brain A β 40 and A β 42 levels in male castrated mice were lower upon treatment with the non-aromatisable dihydrotestosterone (DHT), but were not affected by treatment with estrogen. In addition, high testosterone caused a significant increase in plasma A β 40 levels at days 27 and 36. This could possibly be the result of clearance of the high brain A β 40 levels, yet once in the bloodstream, A β 40 is thought to be rapidly broken down (Hone, 2003). Studies are needed to establish why changes to CSF and A β levels are so different, and importantly, whether the increased levels are the result of improved clearance.

Similarly, brain and plasma levels of A β were found to be affected differently by DHT in experiments by Ramsden *et al.* 2003. Higher doses of most androgens produce more aromatisation, as seen with the supra-physiological levels of testosterone reached with intramuscular injections of testosterone enanthate in studies of cognition in older men. This treatment doubles estradiol levels in moderate doses, and quadruples them with large supra-physiological doses (Cherrier, 2007). Only the moderate dosage group showed improvements in spatial and verbal tests, suggesting an inverted U-shaped response pattern to both testosterone and estrogens. The route of administration of testosterone should also be evaluated, as transdermal testosterone administration (as opposed to intramuscular or by pellet) only raises estradiol levels slightly, while quadrupling DHT levels, due to 5 α reductase enzymes in the skin (Swerdlow, 2000).

The investigation of other APP-related proteins and other hormones and related proteins, such as apolipoprotein E, LRP, sex-hormone binding globulin, free testosterone, estrogen, DHT, AR, FSH and LH is required to obtain a more complete picture of the interactions between components of the hypothalamic-pituitary-gonadal axis, and to characterise better the effects of these interactions on A β metabolism. The next two chapters analyse the effects of one of these components, LH, at modulating A β metabolism in the guinea pig CNS. The results presented in this chapter have highlighted the need to understand the long-term changes brought about by testosterone treatment on components of the hypothalamic-pituitary-gonadal axis, as short-term studies provide only a partial understanding of the dynamics of the system. The results also demonstrate that the measurement of blood plasma A β levels, are not sufficient to monitor the efficacy of androgen therapy for the prevention of AD.

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Chapter 4

CNS administration of human luteinizing hormone increases cerebrospinal fluid and cerebral beta amyloid levels in guinea pigs

4.1 Introduction

Age-associated reductions in sex steroids have been implicated in cognitive impairment, dementia and the pathogenesis of AD. Results presented in Chapter 3 show that reductions in serum testosterone increased CNS levels of A β in the gonadectomised guinea pig, providing further evidence for a role of this sex steroid in AD pathogenesis. However, there is growing evidence to suggest that increased gonadotropin levels during aging may also contribute to AD pathogenesis. Compared to age-matched controls, serum levels of gonadotropins have been shown to increase in subjects with AD (Bowen, 2000; Short, 2001). Furthermore, findings from our group have shown that increased serum LH levels are associated with poorer cognitive performance in post-menopausal women (Rodrigues, 2008) and that serum LH levels are associated with plasma A β 40 levels in cognitively normal elderly men (Verdile, 2008).

Initial evidence that LH may be involved in AD pathogenesis was provided by Bowen and colleagues (2002) who reported an increase in LH immunoreactivity in the pyramidal neurons of the AD brain compared with age-matched controls. Coupled with findings that LH modulates APP processing and A β generation *in vitro*, and that gonadotropin releasing hormone (GnRH) agonists decrease A β levels in mouse brain (Bowen, 2004; Casadesus, 2006), these findings indicate that the increased gonadotropins are a physiologically relevant signal that potentially modulates neurodegeneration in the aging brain.

The mechanisms by which LH modulates A β metabolism remain to be determined. Earlier studies have shown the presence of pituitary and gonadal hormones in the CSF of rabbits (Heller, 1968; Knowles, 1972). More recently a small percentage (<1%) of radio-labelled hCG injected into rats was shown to cross the blood brain barrier into the CSF and was also detected in the hippocampus (Lukacs, 1995). In addition, peripheral administration of the LH analogue, hCG, to rodents, has been shown to impair hippocampal associated behaviours (Barron, 2010; Berry, 2008; Lukacs, 1995). Together, these studies provide evidence that systemic gonadotropins can enter the CNS

and can impact on memory and learning. There is also evidence that LH is synthesized in the brain and components of LH receptor signalling, steroidogenic acute regulatory (StAR) protein and cytochrome P450 side-chain cleavage enzyme (P450scc), have all been identified in neuronal cells (Bowen, 2002; Liu, 2000; Mukai, 2006 ; Murakami, 2006 ; Webber, 2006 ; Wilson, 2006) . The LH receptor itself has been shown to be expressed in neurons from the hippocampus, an area of the brain involved in memory formation and severely affected in AD (Lei, 1993). The existence of LH signalling pathway in neurons is still not well understood. However there is some evidence *in vitro* to suggest that modulation of the neuronal LH receptor can impact on neurosteroidogenesis (Liu, 2007) . Exposure of neuronal cells in culture to LH has been shown to result in an accumulation of A β (Bowen *et al.*, 2004). Further, our group and others have shown that peripheral administration of hCG, to rodents, increases cerebral A β (Barron, 2010 ; Berry, 2008) . However, whether LH directly alters A β metabolism in the CNS, *in vivo*, remains to be determined. This chapter investigates whether direct exposure of LH to the CNS can alter A β metabolism in guinea pig brain.

4.2 Aims

The aims of my study were:

- To determine if the slow release of LH into the guinea pig CNS increased CSF and brain A β 40 levels.
- To determine if increased brain A β 40 levels are associated with changes in levels of APP and its metabolites.

4.3 Methods

Animals

A total of 44 adult male guinea pigs, body weight (BW) 500 gram (\pm 6-8 weeks old) underwent experimentation. For a set of experiments guinea pigs were either gonadectomised (GDX) (n=10) under halothane anaesthesia (3-4% induction and 1-2% maintenance with Halothane) or left non-gonadectomised (non-GDX) (n=10). Another group of animals (n=24) were GDX as above and left for one week prior to implanting slow release pellets (Innovative Research of America, Florida, USA) containing either 0.045 mg (equal to 2.25 μ g/kg BW/day) human LH (MP Biomedicals; n=12) or placebo (n=12) under combination of atropine-diazepam-hypnorm anaesthesia (0.05 mg/kg BW atropine, subcutaneously; 5 mg/kg BW diazepam, intraperitoneally and 1 ml/kg BW hypnorm, intramuscularly). This combination provided deep anaesthesia for about 45 minutes. Once anaesthesia was achieved, guinea pigs were placed in a stereotaxic frame (David Kopf Instruments) and partial craniotomy was performed to expose the underlying dura. The dura was incised and a pellet (5 mm in diameter) was placed in the interhemispheric subdural space of the frontal lobe (stereotaxic coordinate: A 13.0–A 9.0, i.e.: 13 to 9 mm anterior to the frontal zero plane, according to Rapisarda and Bacchelli, 1977 (Rapisarda and Bacchelli, 1977)). After the surgery, the dura was stitched, the cranium was replaced and the skin was sutured. An analgesic, buprenorphine (0.05 mg/kg BW, subcutaneously) was administered and animals were kept warm until they recovered. Animals were sacrificed at 14 or 28 days post-administration of the pellets. Six animals treated with placebo and 6 animals treated with LH were sacrificed at each time point.

CSF, plasma and tissues collection

The CSF, plasma and tissue collection was performed as described in section 2.2.2.2 and 2.2.2.3. Briefly, at each time point, animals were anaesthetised with halothane and blood was collected via cardiac puncture. CSF was collected from the cisterna magna. Processing of whole blood (to collect plasma) and CSF, prior to analysis of A β 40 levels is described in section 2.2.5 To collect brain tissue, all animals were cardiac-perfused

with heparinized-cold phosphate buffer saline (PBS) and the brain removed. The brains were dissected into regions, such as frontal cortex, hippocampus and cerebellum, and homogenized in PBS containing protease inhibitor. Brain homogenates were used for identification for APP metabolites. Full length (FL) APP, and APP-CTF were assessed by western immunoblotting as described in section 2.2.3), A β 40 levels were assessed by ELISA (as described in section 2.2.5).

Measurement of serum testosterone plasma and CSF A β 40 levels.

Serum testosterone was measured in non-GDX and GDX guinea pigs by Path Centre (Perth, Australia) and plasma and CSF A β 40 levels were measured by sandwich ELISA using the monoclonal 6E10 as capture antibody (anti-human A β , Signet Laboratories, USA) and the polyclonal R208 as a detection antibody (kindly provided by Dr. Pankaj Mehta, NYS Institute for Basic Research, New York, USA) (see chapter 2.1.3.2).

Statistical analysis

The student t-test (assuming unequal variance) was used for data comparisons between non-GDX and GDX animals. All other data were analysed using two-way analysis of variance (ANOVA) with LSD Post-hoc test at the 0.05 significance levels.

4.4 Results

4.4.1 CSF and brain A β 40 levels are altered following LH administration to guinea pig CNS.

To determine the direct effects of LH on CNS A β levels, placebo or LH slow release implants were administered to the interhemispheric subdural space of the frontal lobe. As the guinea pigs were GDX prior to pellet implantation, the effect of GDX on CSF, plasma and cerebral A β 40 levels were assessed in the GDX animals compared to non-GDX control animals.

As expected GDX led to a reduction in testosterone levels (non-GDX animals = 28.65 ± 6.22 ; GDX 3.7 ± 0.62 nmol/L, $p < 0.001$). Analysis of CSF A β 40 levels revealed that GDX resulted in significant increase in CSF A β 40 levels compared to the non-GDX control animals ($p < 0.05$; Figure 4.1A). The addition of the LH implant increased CSF A β 40 levels further. Two-way analysis of variance (ANOVA) showed that the LH treatment ($F = 34.453$; $p < 0.001$) and the duration of treatment ($F = 26.237$; $p < 0.001$) both significantly impacted on CSF A β 40 levels. An interaction between the LH treatment and duration of treatment was also significant ($F = 7.692$; $p < 0.05$). Post-hoc analysis revealed that there was a significant increase in CSF A β 40 levels following 14 ($p < 0.05$) and 28 days ($p < 0.001$) of LH treatment (Figure 4.1B). Overall, these results indicate that although GDX led to an increase in CSF A β 40 levels, the addition of exogenous LH led to a further increase, which was dependent on duration of treatment.

I next determined if these effects extended to the periphery by measuring plasma A β 40 levels from non-GDX, GDX or LH or placebo treated animals. Compared to non-GDX animals, GDX did not alter A β 40 levels (Figure 4.2A). Similarly, compared to animals administered with placebo, plasma A β 40 levels were not altered in those administered LH (Figure 2B).

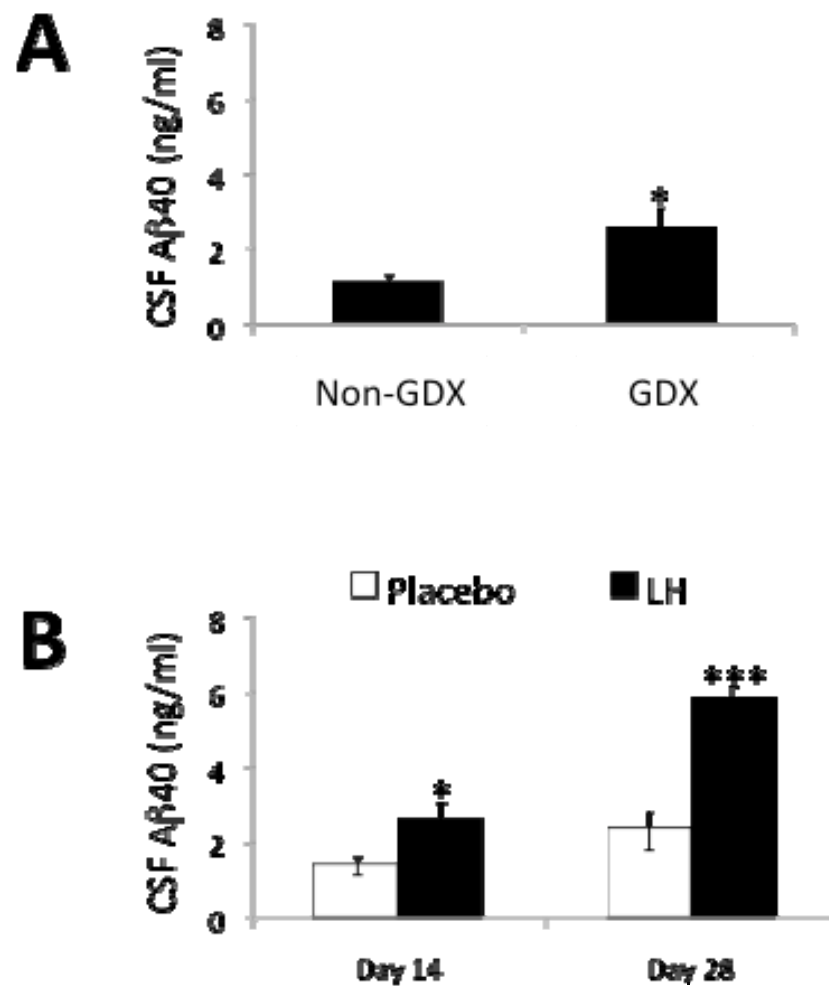


Figure 4.1: CSF Aβ40 levels from non-GDX, GDX or GDX guinea pigs administered placebo or LH for 14 or 28 days.

Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 14 or 28 and CSF was collected from the cisterna magna. (A) GDX resulted in increased levels of CSF Aβ40 compared to the non-GDX control animals (* $p < 0.05$). (B) CSF Aβ40 levels were significantly increased in animals administered LH at 14 and 28 days post-treatment compared to guinea pigs administered placebo (* $p < 0.05$, *** $p < 0.001$). All data are shown in Mean \pm SEM.

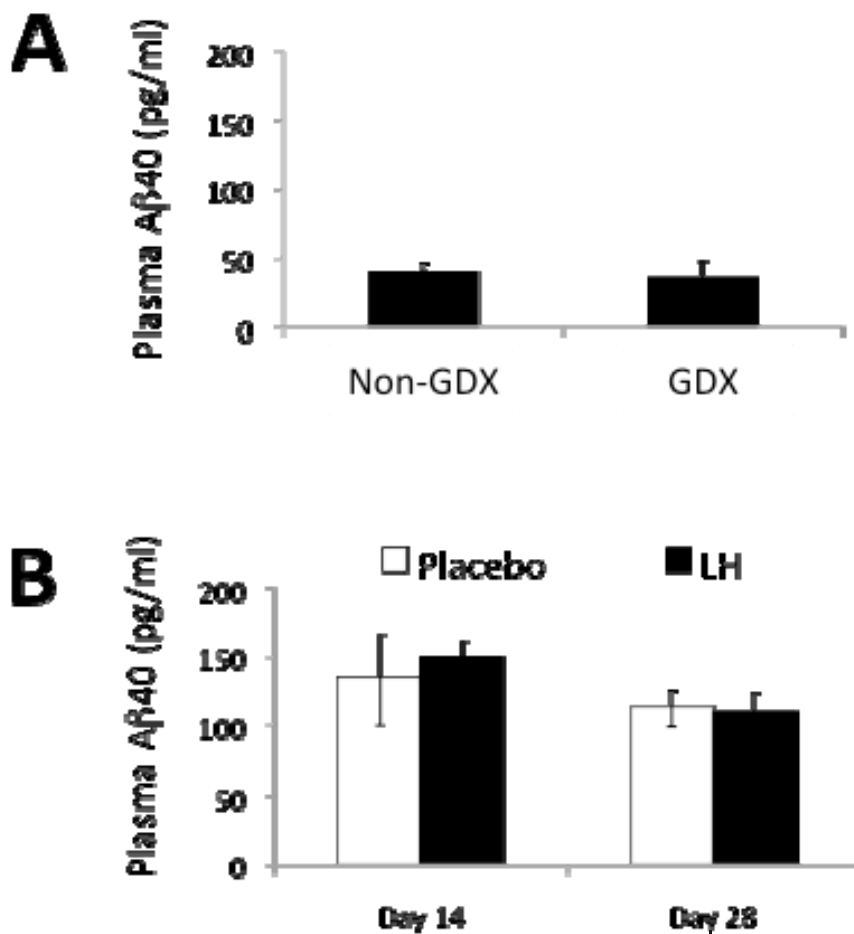


Figure 4.2: Plasma Aβ40 levels from non-GDX, GDX or GDX guinea pigs administered placebo or LH for 14 or 28 days.

Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 14 or 28 and plasma was collected. (A) Plasma Aβ40 levels were not altered as a result of GDX (B) No change in plasma Aβ40 levels were observed in animals administered placebo or LH. All data are shown in Mean ± SEM.

Having shown that GDX and the direct cerebral exposure to LH resulted in increased Aβ40 levels in CSF, I next investigated whether brain Aβ40 levels were altered in these animals. The frontal cortex and hippocampus were isolated and underwent analysis for Aβ40. The Aβ40 levels within the frontal cortex of GDX animals were similar to those

observed for non-GDX animals (Figure 4.3A). Administering the LH implant resulted in an increase in A β 40 levels within the frontal cortex. Two-way analysis of variance (ANOVA) showed that there was a main effect of LH treatment ($F=12.171$, $p<0.01$; Figure 4.3B), however there was no effect of duration of treatment ($F=0.789$, $p<0.385$). *Post hoc* analysis revealed that A β 40 levels were significantly increased following 28 days ($p<0.05$), although a trend towards an increase was observed following 14 days of treatment.

In contrast to A β 40 levels in the frontal cortex, those observed in the hippocampus of GDX animals were significantly increased compared to levels from hippocampus of non-GDX animals ($p<0.01$; Figure 4.4A). The addition of LH resulted in a further increase in A β 40 levels within the hippocampus. Two-way analysis of variance (ANOVA) exhibited similar findings to that observed in the frontal cortex, there was a main effect of LH treatment ($F=12.116$, $p<0.01$; Figure 4.4B). However there was no effect of duration of treatment ($F=0.478$, $p<0.499$). *Post-hoc* analysis revealed that compared to placebo treated animals, hippocampal A β 40 levels were significantly increased following 14 or 28 days treatment with A β ($p<0.05$). Overall the results indicate that the addition of exogenous LH, results in an increase in cerebral A β 40 levels.

To determine if the increase in A β 40 levels observed with LH was due to altered APP processing, brain homogenates underwent western immunoblotting for FL-APP and its C-terminal fragments (APP-CTF). In frontal cortex tissue, compared with placebo, no significant changes were observed in FL-APP and APP-CTF following treatment with LH (Figure 4.5A). Analysis of hippocampal tissue (Figure 4.5B) revealed a main effect of LH treatment on FL-APP ($F=6.762$, $p<0.05$) and APP-CTF ($F=9.06$, $p<0.01$) levels, this was not dependent on duration of treatment. *Post-hoc* analysis revealed that FL-APP and APP-CTF levels were significantly increased following 28 days of treatment ($p<0.05$). The LH associated increase in APP-FL and APP-CTF levels in the hippocampal tissue, may explain the increased A β 40 levels observed in this brain region.

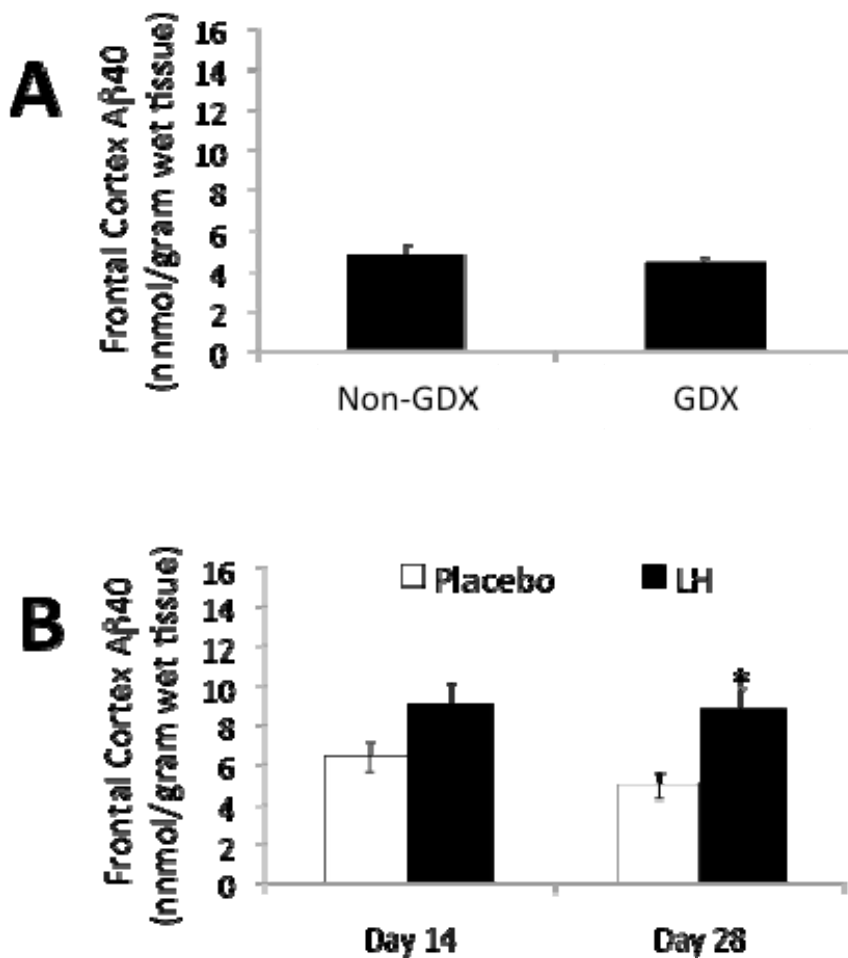


Figure 4.3: Aβ40 levels from frontal cortex of non-GDX, GDX or GDX guinea pigs administered placebo or LH for 14 or 28 days.

Cerebral Aβ40 levels were measured in frontal cortex of non-GDX control, GDX animals and animals administered placebo or LH slow release pellets. (A) There were no different in frontal cortex Aβ40 levels between non-GDX control and GDX animals. (B) Compared to placebo treated animals, a trend towards an increase in Aβ40 levels were observed in the animals treated with LH for 14 days. Following 28 days of treatment, Aβ40 levels in the frontal cortex were significantly increased in LH treated animals (* $p < 0.05$). All data are shown in Mean \pm SEM.

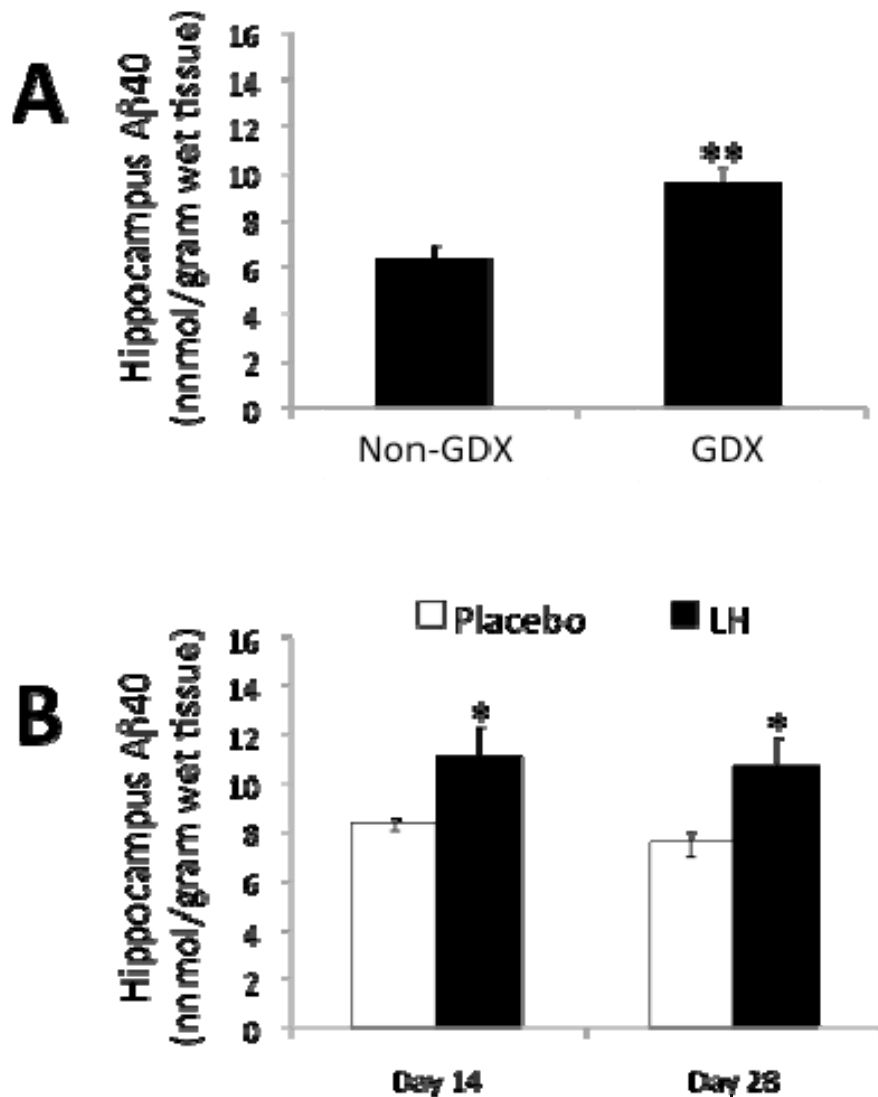
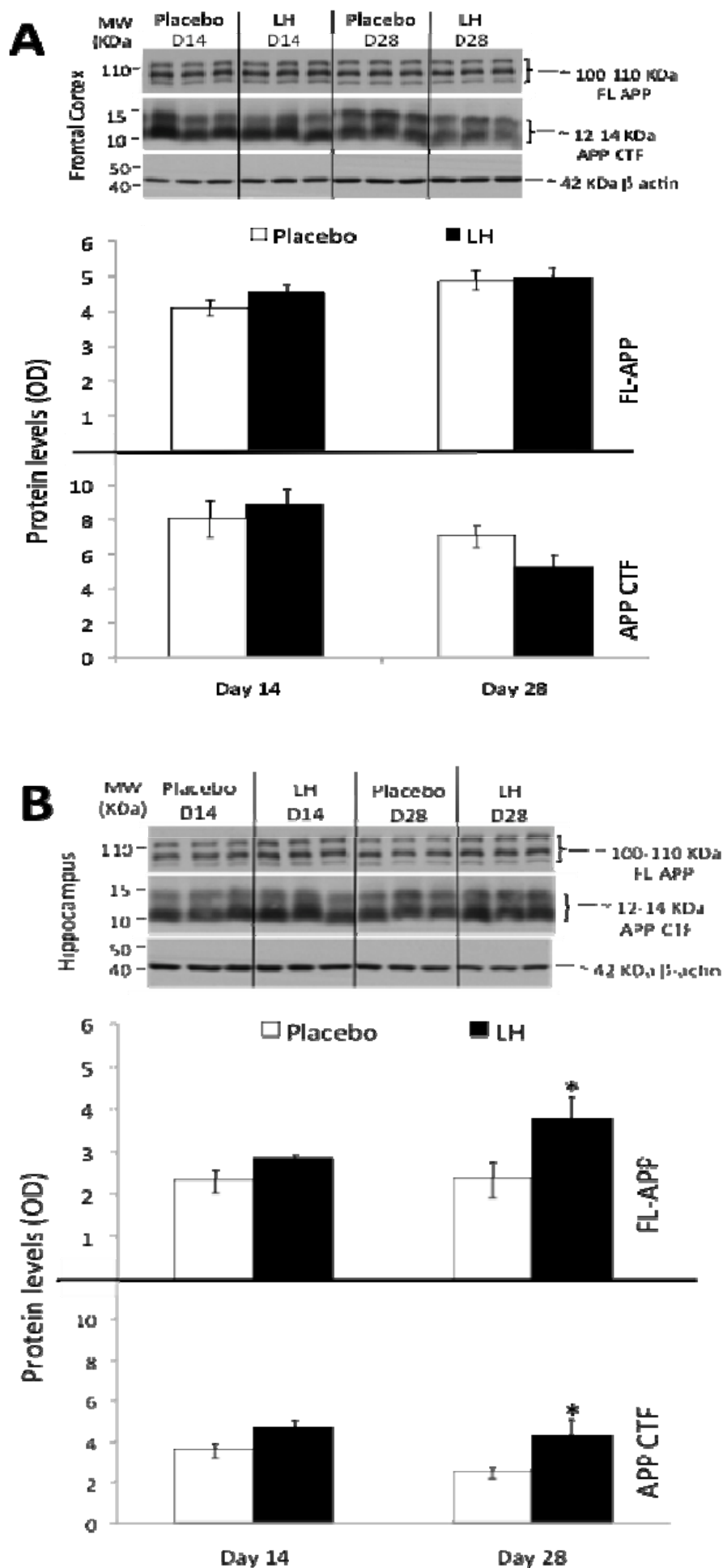


Figure 4.4: A β 40 levels from hippocampus of non-GDX, GDX or GDX guinea pigs administered placebo or LH for 14 or 28 days.

(A) Gonadectomy (GDX) resulted in increased levels of hippocampus A β 40 levels in GDX animals compared to non-GDX control animals (** $p < 0.01$). (B) A significant increased in hippocampal A β 40 levels were observed in those animals treated with LH at day 14 and day 28 compared to non-GDX control animals (* $p < 0.05$). All data are shown in Mean \pm SEM.

Figure 4.5: Levels of full length APP and its C-terminal fragment in frontal cortex and hippocampus of GDX guinea pigs administered placebo or LH for 14 or 28 days.

(A) Compared to GDX animals administered placebo, there were no significant changes observed in FL-APP and APP-CTF levels as a result of LH supplementation at 14 and 28 days LH pellet administration. (B) Hippocampal levels of FL-APP and APP-CTF levels were significantly increased in homogenates of animals following 28 days of LH treatments (* $p < 0.05$). All data are shown in Mean \pm SEM.



4.4.2 Increased brain P450scc protein levels following administration of LH to guinea pig.

Our results above indicate that cerebral A β levels were significantly altered in the frontal cortex and hippocampus of guinea pigs administered LH. We next examined expression levels of the P450scc enzyme, in the frontal cortex and hippocampus, as an indicator of the activation of LH signalling. The results show expression of the ~ 49 kDa P450scc enzyme in both brain regions (Figure 4.7A). A similar sized protein band (49-50 kDa) has been reported in rodent brain (Chia, 2008 ;Kimoto, 2001) . Analysis of levels of P450scc in the frontal cortex (Figure 4.6A) and hippocampus (Figure 4.6B) revealed a main effect of LH treatment ($F=16.647$, $p<0.01$ and $F=16.688$, $p<0.01$, respectively) where significant increases were observed at day 28 ($p<0.01$). Unexpectedly, the levels of P450scc in the hippocampus of the placebo groups were also significantly decreased from day 14 to day 28 ($p<0.05$). The experiment was performed three times and a similar result was achieved on each occasion. Further, β -actin levels were shown to be similar in all samples, ruling out the possibility of sample loading errors. To investigate this further and determine if the dramatic reduction was due to GDX, another aliquot of hippocampal homogenates from non-GDX, GDX or GDX animals containing placebo implants underwent western immunoblotting for P450scc. The results show that compared to non-GDX control guinea pigs, levels of P450 were significantly decreased in GDX or GDX animals implanted with placebo pellets (Figure 4.6C), indicating that GDX impacted on hippocampal expression of P450scc.

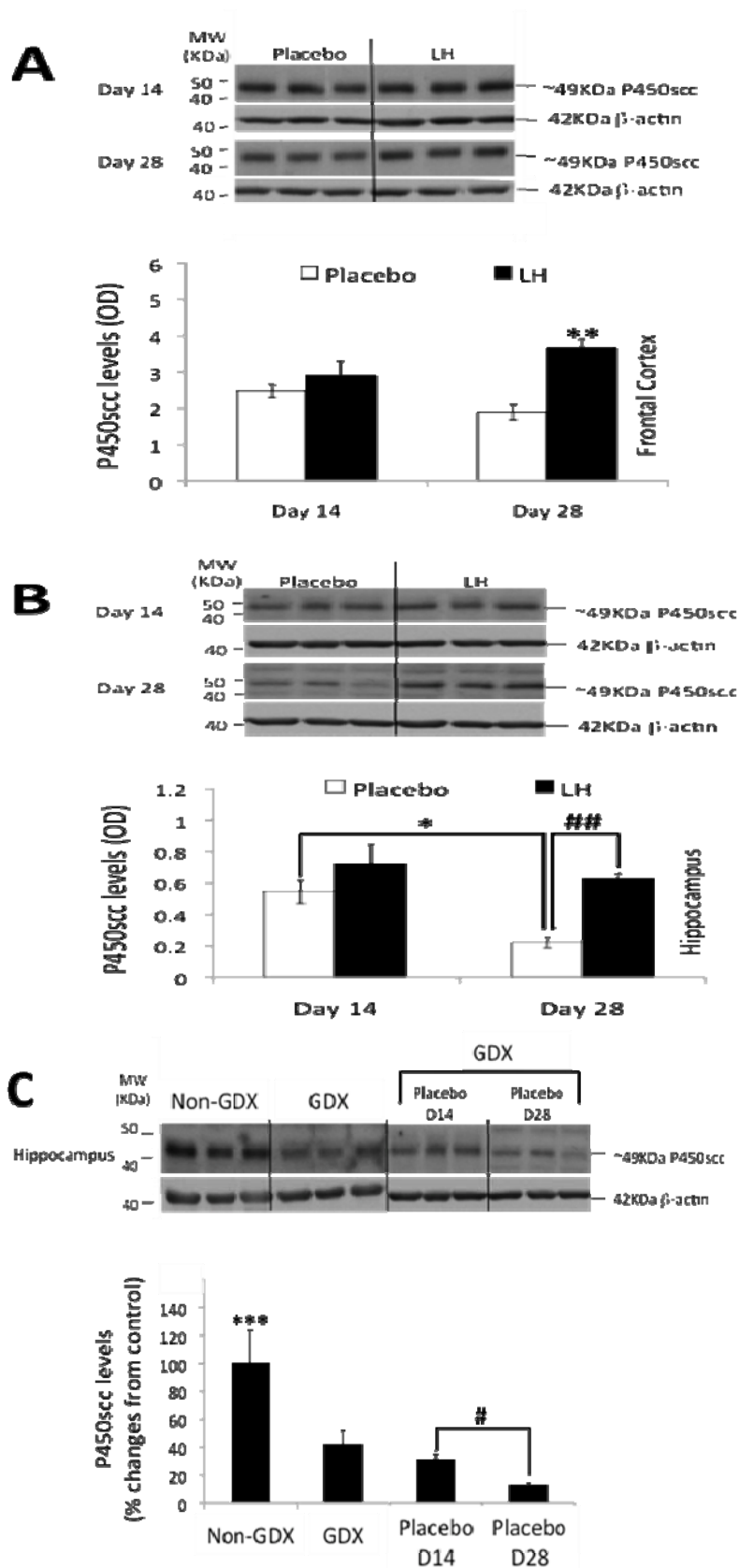


Figure 4.6: Levels of cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) in frontal cortex and hippocampus of non-GDX, GDX or GDX guinea pigs administered placebo or LH for 14 or 28 days.

(A) Compared to placebo treated animals, P450scc levels within the frontal cortex were significantly increased following 28 days of LH supplementation (** $p < 0.01$). (B) In the hippocampus, P450scc levels were also increased significantly at 28 days post LH treatment (## $p < 0.01$). Unexpectedly, the levels of P450scc in the hippocampus of the placebo groups were significantly decreased from day 14 to day 28 (* $p < 0.05$). (C) Hippocampal levels of P450scc were measured in homogenate from non-GDX, GDX and GDX animals administered placebo for 14 or 28 days. Compared to the non-GDX control animals, P450scc levels were significantly reduced in all GDX animals (** $p < 0.001$). Compared to animals administered placebo for 14 days, hippocampal levels of P450scc were significantly reduced in those animals treated for 28 days (# $p < 0.05$). All data are shown as the Mean \pm SEM.

4.5 Discussion

Evidence supports the notion that LH promotes the amyloidogenic pathway (Bowen, 2004), and that peripheral administration of its analogue, hCG can impair memory and learning in rodents and result in the accumulation of cerebral A β levels (Barron, 2010; Berry, 2008). However, there is yet no *in vivo* data to indicate that the direct application of LH itself to the CNS can alter A β metabolism. This chapter addressed this by implanting slow release pellets in the parietal cortex of the GDX guinea pig brain.

We have previously shown that plasma and CSF A β 40 levels were increased following GDX of male guinea pigs (Chapter 3, Wahjoepramono, 2008). Although in the current study plasma A β 40 levels were unaltered [possibly due to the shorter period of GDX in this study (14 days) compared with that of [Chapter 3, Wahjoepramono, 2008 (36 days)], increased CSF A β 40 levels were observed. We have extended these findings to also show increased cerebral A β levels.

However, analysis of A β 40 levels in the frontal cortex and hippocampus revealed contrasting results. GDX did not alter levels of A β 40 within the frontal cortex, whereas an increase in A β 40 levels was observed in the hippocampus. Immunohistochemical analysis of brain tissue from GDX male transgenic mice has shown marked deposition of A β in the subiculum and the CA1 region of the hippocampus (Rosario, 2010; Rosario, 2006). However, these studies did not report any changes within the frontal cortex. The availability of larger quantities of brain tissue from guinea pigs has provided the opportunity to undertake a quantitative analysis of endogenous A β and indicates that there are regional specific changes in A β levels associated with gonadectomy. Reductions in testosterone as a result of GDX would also lead to a corresponding increase in peripheral LH levels and evidence exists that this increase may impact on the hippocampal associated functions. A small percentage of peripheral

gonadotropins can cross the BBB and impact on hippocampal dependent memory and behaviour in rodents (Heller, 1968 ;Knowles, 1972;Lukacs, 1995) . Further, peripheral administration of the more potent analogue of LH, hCG has been shown to impair working memory in rodents and increase in cerebral A β levels (Barron, 2010 ;Berry, 2008) . Studies also suggest that LH is localised to the hippocampus and is the most likely site of LH signalling in the brain (Bowen, 2002;Liu, 2007 ;Wilson, 2006) . Together, these findings indicate that the hippocampus may be a major target for gonadotropins within the brain.

Further evidence for this notion has been presented in the findings in the current chapter where the addition of exogenous LH, led to differences in levels of A β in the hippocampus and the frontal cortex. Significant increases in A β 40 levels were observed in the hippocampus earlier (at day 14- post implantation) compared to the increases observed in the frontal cortex. Further, there was a significant increase in expression levels of APP and its C-terminal fragment in the hippocampus as a result of LH treatment. This increase was not observed in the frontal cortex. Increases in APP expression have been previously reported when human embryonic stem cells are treated with hCG (Porayette, 2007) , and the amyloidogenic and non-amyloidogenic processing of APP have been shown to drive these cells into cell proliferation or differentiation into neuronal precursor cells (Porayette, 2009) . In results presented in Figure 4.5, the increase in APP and its fragments within the hippocampus most likely accounts for the increase in A β 40 levels observed in this region of the brain.

Analysis of CSF showed that both LH and duration of treatment impacted on A β 40 levels, where there was a significant increase following 14 and 28 days treatment. No changes were observed in plasma A β 40 levels indicating impaired efflux of A β from the CNS, resulting in the accumulation of A β within the CNS. A β is known to cross the BBB into the periphery through a number mechanisms and impaired efflux of A β from the CNS is one mechanism thought to contribute to the accumulation of A β in the brain [reviewed in (Bates, 2009)]. In chapter 3, data was presented to show that administering testosterone to GDX guinea pigs results in a reduction in CSF A β levels

and an increase in plasma A β levels, suggesting that testosterone may promote clearance of A β from the CNS (Wahjoepramono, 2008).

Although no significant changes in the brain apoE levels with LH administration (data not shown) was observed,, investigating the effects of LH on other transport proteins involved in transporting A β across the BBB, e.g. RAGE, LRP-1, A β degradative enzymes or microglia would be of interest to confirm and provide further insight into mechanisms involved in the apparent impairment in A β clearance from the CNS.

The results presented in this chapter also show for the first time, *in vivo*, that the addition of human LH increases the expression of endogenous brain levels of P450scc. In gonadal tissues P450scc converts cholesterol to the steroid precursor, pregnenolone, and it is up-regulated when the LHR signalling pathway is activated *in vivo* (Scott, 1990) and (Luo, 2005) ;Rekawiecki, 2005) . Components of the LHR signalling pathway have been shown to be present in neuronal cells (Bowen, 2002 ;Liu, 2007 ;Mukai, 2006 ;Murakami, 2006 ;Wilson, 2006) and in rodent brain (Kimoto, 2001). While assessing levels of other components in the LH signalling pathway such as StAR, pregnenolone or other neurosteroids would provide further evidence of activation of LH signalling, it is probable that the increased levels of P450scc in the frontal cortex reflect activation of LH signalling.

It is noted, however, that there was a dramatic decrease in P450scc levels in the hippocampus of animals treated with placebo for 28 days compared to 14-day treatment. This result was unexpected and the reason for the marked reduction is unclear. However, compared to non-GDX animals, a similar reduction in P450scc levels was observed in the hippocampus of GDX animals (which did not undergo surgical placement of implant), ruling out the possibility that this was an artefact of the implant itself.

This reduction may reflect a down regulation of P450scc synthesis due to prolonged period of testosterone depletion. While, there is no current evidence for this occurring in the brain, age related reductions in testosterone in rat leydig cells have been shown to correlate with reductions in P450scc and StAR mRNA transcript and protein levels (Luo, 2005) . Although this reduction was observed over months in the Luo *et al.*,2005 study, down-regulation of P450scc may be accelerated under conditions of testosterone depletion. Despite this reduction, the addition of LH resulted in an up-regulation of P450scc expression, but whether this increase was sustained over a longer period was not addressed in this study.

Although our results show an LH-mediated up-regulation of P450scc, whether increases in A β levels were associated with activation of LH signalling was not addressed in the current study. However, findings from a recent study, suggest that the signalling pathway may play an important role in A β accumulation (Lin, 2010) . In this study, crossing of LHR-/- mice with the AD transgenic model Tg2576 resulted in a reduction in AD pathology, namely amyloid deposition, astrogliosis and tau phosphorylation (Lin, 2010). Interestingly, while LHR-/- mice are devoid of sex steroids for the duration of life and show increased peripheral levels of gonadotropins, depletion of sex steroids and increased accumulation of gonadotropins has previously been associated with exacerbated AD pathology (Bowen, 2002 ;Rosario, 2006) .

While it is difficult to explain the reason(s) why ablation of LHR prevents A β deposition yet leads to other conditions normally associated with increased AD pathology, the study by Lin *et al* (Lin, 2010) is the first to implicate LHR in AD pathogenesis and provides evidence that LH signalling pathways play an important role in A β metabolism and accumulation. Studies using cell culture models utilising various LH receptor binding or signalling mutants may address some of the mechanisms associated with LH mediated modulation of A β metabolism (Lee, 2002) . However, it should also be noted that LH could act through receptor independent mechanisms as is evident in findings that have shown gonadotropin-mediated effects on non-gonadal tissue, when LH receptor is absent, in low abundance or dysfunctional (Yarram, 134

2003;Allan, 2010) . Overall the findings from my study have provided initial evidence that direct exposure of the CNS to LH can alter A β metabolism. Whether this occurs at more physiological concentrations of LH, is addressed in the next chapter where LH or the GnRH agonist (leuprolide acetate) that has been shown to lower endogenous LH, have been added systemically to the guinea pig.

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Chapter 5

Peripheral administration of human luteinizing hormone (LH) and leuprolide to the guinea pig impacts on CNS A β levels.

5.1 Introduction

As described in detail in Chapters 1 and 4, increases in gonadotropin levels, particularly, luteinizing hormone (LH) during aging, may contribute to AD pathogenesis (Bowen *et al.*, 2004; Casadesus *et al.*, 2006, 2007, Webber *et al.*, 2007, Verdile *et al.*, 2008, Berry *et al.*, 2008, Barron *et al.*, 2010, Wahjoepramono *et al.*, 2011). There is accumulating evidence (both *in vitro* and *in vivo*) in support of exogenous human LH (Bowen *et al.*, 2004; Casadesus *et al.*, 2006; Wahjoepramono *et al.*, 2011), over-expression of LH (Casadesus *et al.*, 2007) or addition of its more potent analogue, hCG (Barron *et al.*, 2010; Berry *et al.*, 2008; Lucaks *et al.*, 2005) can modulate accumulation of cerebral A β levels and impair hippocampal associated behaviours. In the previous chapter, the direct application of LH to the guinea pig CNS was shown to increase CSF and cerebral A β levels. This chapter investigated whether peripheral administration of LH can modulate CNS levels of A β . It also assesses the efficacy of the GnRH agonist, leuprolide at modulating CNS A β levels in the guinea pig.

Leuprolide (Leuprolide acetate) is currently used for the treatment of endometriosis (Strowitzki *et al.*, 2012), prostatic cancer (Trachtenberg, J. 1983; N.J. Wojciechowski, 1986; Leonard, S.M, 2003, and precocious puberty (Kappy *et al.*, 1989 ; Neely *et al.*, 1992; Clemons *et al.*, 1993). Both GnRH agonists or antagonists are analogues designed by specific amino acid substitutions to alter efficacy, affinity and prolong their duration of action (reviewed by Millar *et al.*, 2004). GnRH has a half life of 2-4 minutes (Jeffcoate *et al.*, 1974) and its analogues have a half life in the range of hours. Administration of the agonist, leuprolide, results in an initial increase or “flare effects” in serum gonadotropin secretion for several days which is followed by a precipitous reduction. This decrease is a result from the loss of GnRH signalling due to the desensitization of pituitary GnRH receptors which will lead to downregulation of gonadotropin secretion (Periti *et al.*, 2002).

Administering leuprolide to wild type or APP transgenic mice has been shown to reduce brain A β levels and deposition (Bowen *et al.*, 2004; Casadesus *et al.*, 2006). The use of a larger, non-transgenic model such as the guinea pig allows regional differences in

soluble brain A β levels and effects on CSF A β levels to be analysed. In addition, the absence of APP over-expression and robust amyloid deposition, which may mask the potency of leuprolide at reducing soluble A β levels, will be analysed in a more relevant animal model.

5.2 Aims

- To determine the effects of peripherally administering human LH to guinea pigs on CSF A β , and levels of brain A β , APP and its metabolites.
- To determine if administering leuprolide, reduces CSF and brain A β levels.

5.3 Methods

Animals

Peripheral administration of human LH and Leuprolide to guinea pigs

A total of 36 guinea pigs were gonadectomised (GDX) under halothane anaesthesia as previously described in Chapter 2.2.2.1. One week following GDX, forty-day slow release pellets: placebo, LH, and leuprolide (Innovative Research of America, Florida, USA) were implanted in the periphery (subcutaneous insertion into the dorsal neck fat pad of guinea pigs), as described in section 2.2.2.1. The groups and concentrations of active ingredients within the slow release pellets are shown in Figure 5.1. **Note** that, compared to animals that had undergone GDX only (for period of 18 or 36 days), those administered slow release implants had undergone GDX 1 week prior to insertion of implants. Thus, the total time in which guinea pigs exhibited reduced testosterone levels was 25 and 43 days respectively.

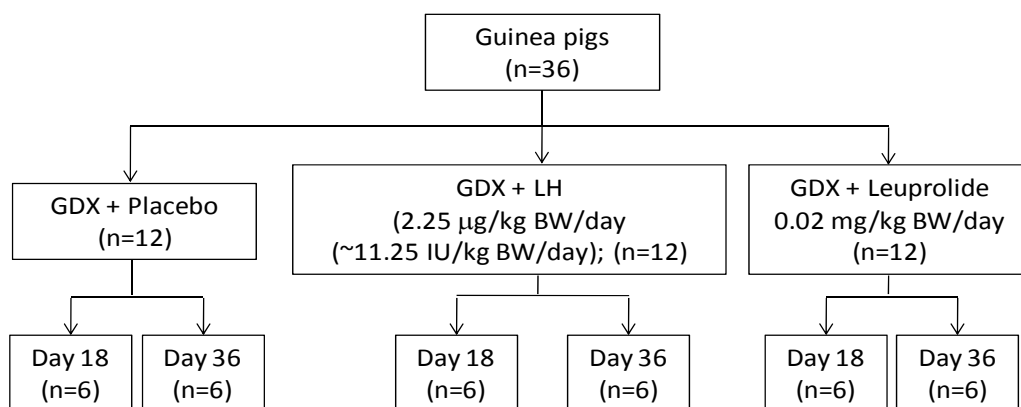


Figure 5.1: A summary of animal groups and treatments used.

CSF and tissue collection

At day 18 or 36 post-implant, animals were sacrificed and CSF and brain were collected to measure the A β 40 levels, as previously described in chapter 2. All animals were cardiac-perfused with heparinised-cold PBS. The brain was removed and snap frozen in liquid N₂ for ELISA measurement of A β 40. Prior to each assay, the brain was dissected into frontal cortex and hippocampus and was homogenized in cold PBS with protease inhibitor. APP and its metabolites were examined semi-quantitatively via western blot as described in chapter 2.2.3. β -actin was used as a loading control protein. A sensitive double-antibody sandwich ELISA assay was used for the detection and measurement of A β levels in tissue homogenates, plasma, and CSF respectively as described in chapter 2.2.5. CSF A β levels used in this Chapter and the previous two Chapters were all measured in the same assay together.

Statistical analysis

Data was analysed using the Statistical Package for Social Sciences (SPSS : version 17; SPSS Inc., Chicago, Illinois, USA). Experiments were 2x2 factorial designs (presence or absence of placebo x presence or absence of LH and/or Leuprolide). Analysis of data were performed by two-way ANOVA at the 0.05 significance level to test for treatment interactions and main effects. If the data was found to be heterogenous, analysis was performed using non-parametric Kruskal Wallis analysis of variance. This is noted in the relevant figure legends below.

5.4 Results

5.4.1 Guinea pig CSF and brain A β levels are altered following peripheral administration of LH.

To determine the effects of the peripheral addition of exogenous LH on CNS A β levels, slow release implants of LH or placebo were administered subcutaneously to gonadectomised (GDX) guinea pigs for 18 and 36 days. A β 40 levels were measured in the CSF, frontal cortex and hippocampus and results are shown in Figures 5.2, 5.3 and 5.4 respectively. As described in chapter 3, (section 3.4.1), compared to intact controls (non-GDX), GDX for 18 or 36 days led to a significant increase in CSF A β 40 levels (Figure 5.2A). This most likely reflects the reductions in testosterone in these GDX animals (see chapter 3, Figure 3.2). Administering exogenous LH led to a further increase (Figure 5.2B). Two-way analysis of variance (ANOVA) showed that there was a main effect of LH ($F=7.934$, $p<0.01$) and an effect on the duration of treatment ($F=4.745$, $p<0.04$) (Figure 5.1B). Pairwise comparison analysis revealed that compared to placebo treated animals, CSF A β 40 levels were significantly increased following 36 days of treatment with LH ($p<0.05$). Overall, these results indicate that although GDX led to an increase in CSF A β 40 levels, the addition of exogenous LH led to a further increase, which was dependent on duration of treatment.

To determine if the effects of LH in the CSF reflected that occurring in the brain the frontal cortex and hippocampus were isolated and underwent analysis for A β 40. As reported in chapter 3, 18 days of GDX did not significantly alter A β 40 levels in the frontal cortex; however levels were significantly reduced following 36 days GDX (Figure 5.2A). Administering the LH implant resulted in a reduction in A β 40 levels within the frontal cortex. Two-way analysis of variance (ANOVA) showed that there was a main effect of LH treatment ($F=35.645$, $p<0.01$; Figure 5.2B) and duration of treatment ($F=5.514$, $p<0.029$), where A β 40 levels were significantly reduced following 18 and 36 days of treatment ($p<0.05$).

In the hippocampus, GDX led to a significant increase in A β 40 levels (Figure 5.3A). This effect of GDX was lost at day 36. In contrast, administering LH led to a significant reduction in A β 40 levels. Two-way ANOVA revealed that there was a main

effect of LH treatment ($F=14.801$, $p<0.001$; Figure 5.3B), however there was no effect of duration of treatment ($F=0.789$, $p<0.385$). These results indicate that the addition of exogenous LH peripherally, negated the effects of GDX on brain A β 40 levels.

To determine if administering LH altered the levels of APP and its metabolites, brain homogenates underwent western immunoblotting for FL-APP and its C-terminal fragments (APP-CTF). Analysis of FL-APP levels in the frontal cortex revealed no significant impact of treatment ($F=0.896$, $p<0.356$) or duration of treatment ($F=0.033$, $p<0.858$). The data for APP-CTF levels was not normally distributed and as such two-way analysis (for normally distributed data) could not be performed. Instead the non parametric, Kruskal Wallis analysis was performed. Analysis revealed no statistically significant changes in APP-CTF. Similar results were observed in hippocampal tissue. Overall, the results indicate that changes observed in cerebral A β 40 levels due to LH administration were not attributed to altered levels of APP and its C-terminal fragment.

To further determine if the reductions in cerebral A β 40 levels were associated with changes in APP metabolism, levels of α -APPs, were measured in the frontal cortex. As mentioned in Chapter 3, this region provides sufficient material for ultracentrifugation to obtain supernatant free of cell debris (as described in section 2.2.2.3). Following A β analysis via ELISA and western blot analysis of APP, the frontal cortex was the only region to have sufficient amount of tissue remaining required to perform this analysis. The sAPP α protein was detected via western immunoblotting and underwent quantitative analysis (Figure 5.6). Analysis by two-way ANOVA showed that there is a main effect of treatment ($F=8.251$, $p<0.014$) and duration of treatment duration of treatment ($F=6.126$, $p<0.029$). Pairwise comparison analysis showed that compared with placebo, α -APPs levels were significantly increased at day 36 ($p<0.05$).

Taken together the results indicate that administering LH peripherally, led to increased CSFA β 40, a similar trend to that seen following direct exposure of LH to the brain (see Chapter 4, Figure 4.1). However, unlike cerebral exposure to LH, administering the hormone, peripherally led to a reduction in A β levels in the frontal cortex and hippocampus. The reduction in A β 40 levels corresponded with an increase in α -APPs in the frontal cortex.

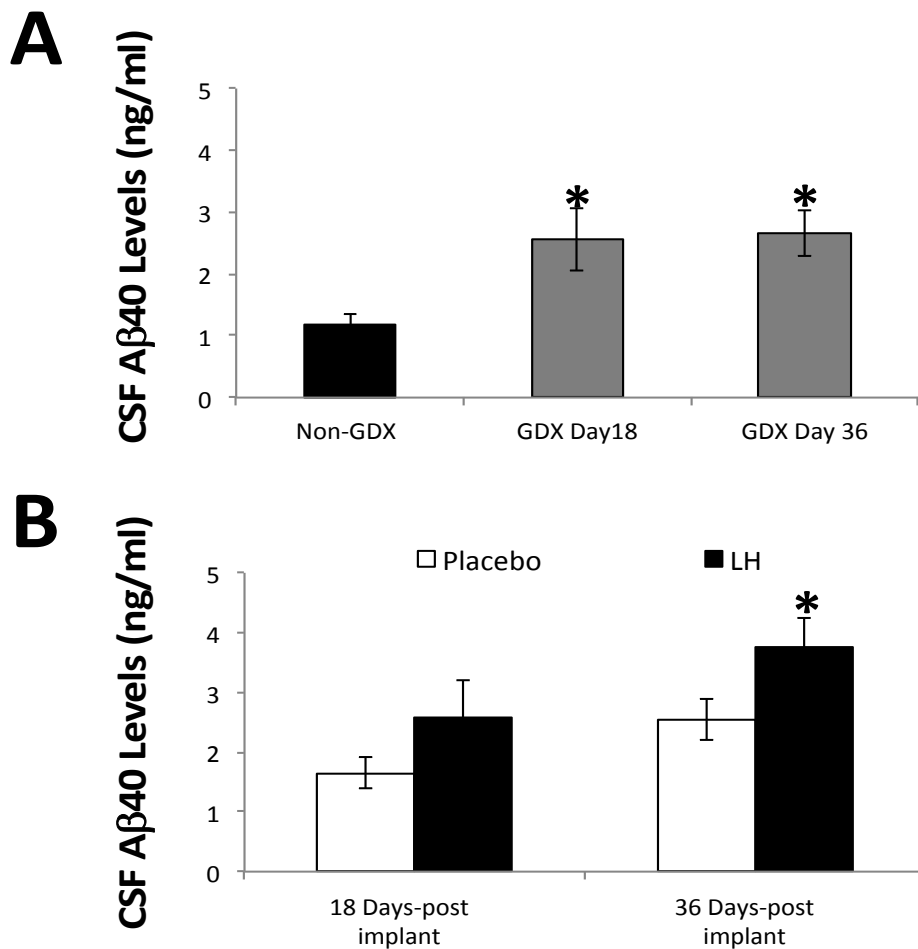


Fig 5.2: CSF A β 40 levels from GDX guinea pigs administered placebo or LH for 18 or 36 days. Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 18 or 36 days and CSF was collected from the cisterna magna.

(A) GDX guinea pigs show increased levels of the CSF A β 40 compared to non-GDX at day 18 and 36 following surgery. Data is represented as mean \pm SEM of 6 animals/group. *, $p < 0.05$. Values significantly increased from non-GDX.

(B) Compared to the placebo group within the same day of treatment, CSF A β 40 was significantly increased in animals administered LH at day 36. Data is represented mean \pm SEM. For placebo day 18, $n=5$, for placebo day 36 $n=8$, for LH day 18 and 36 $n=6$ for each time point. *, $p < 0.05$. Values significantly increased compared to placebo at Day 36.

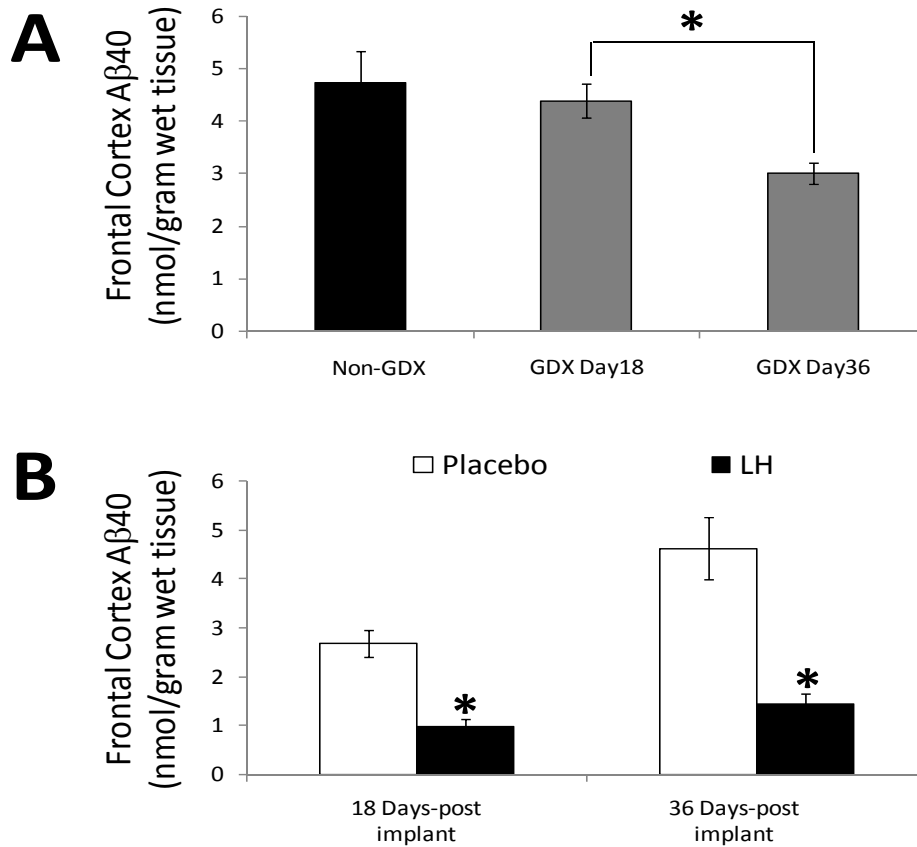


Figure 5.3: Aβ40 levels from frontal cortex of GDX guinea pigs administered placebo or LH for 18 or 36 days. Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 18 or 36 days and Aβ40 levels were measured in the frontal cortex.

(A) GDX guinea pigs show increased levels of Aβ40 compared to non-GDX at day 18 and 36 following surgery. Data is represented as mean± SEM of 6 animals/ group. *, $p < 0.05$, Values significantly reduced compared to GDX at Day 18.

(B) Administering the LH implant resulted in a reduction in Aβ40 levels within the frontal cortex. Two-way ANOVA and pairwise comparison analysis revealed that compared to placebo treated animals, Aβ40 levels were significantly reduced following 18 or 36 days of LH treatment ($p < 0.05$). Data is represented mean± SEM. For placebo day 18, $n=5$, for placebo day 36, $n=8$, for LH day 18 and 36 $n=4$ for each group*, $p < 0.05$. Values were significantly reduced compared to placebo treated animals at both day 18 and Day 36.

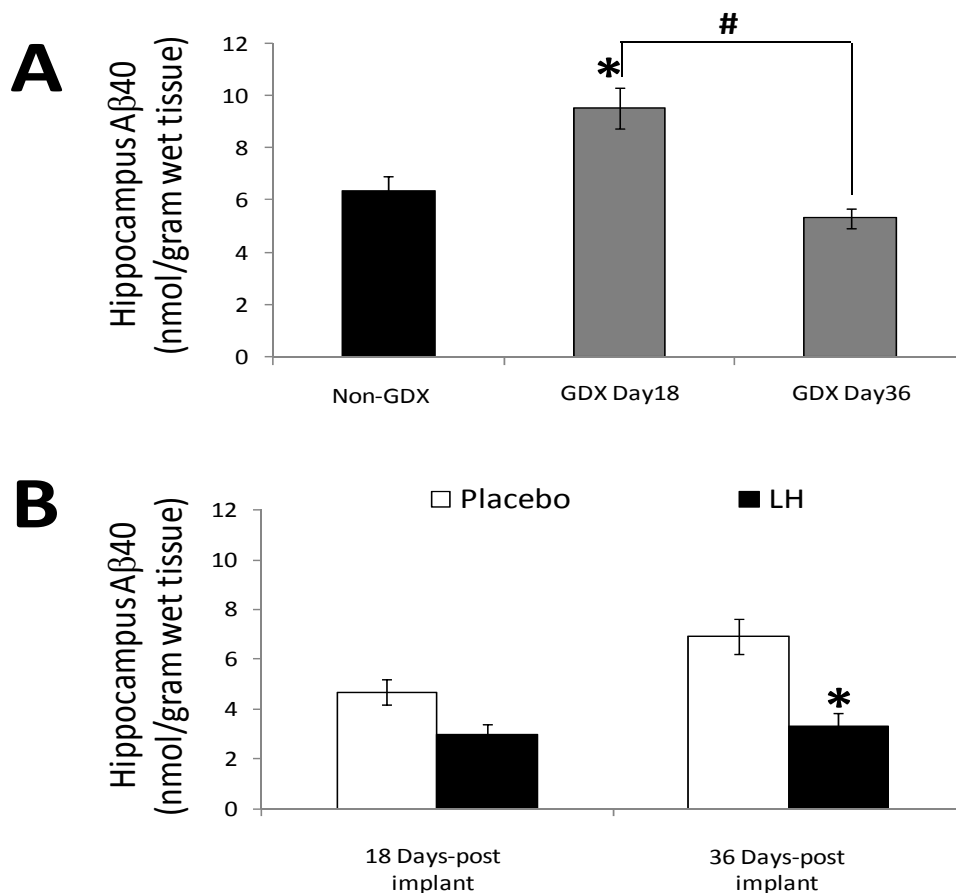


Figure 5.4: Aβ40 levels from hippocampus of GDX guinea pigs administered placebo or LH for 18 or 36 days. Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 18 or 36 days and Aβ40 levels were measured in the hippocampus.

(A) GDX guinea pigs show increased levels of Aβ40 compared to non-GDX at day 18 and but not at day 36 following surgery. Data is represented as mean± SEM of 6 animals per group. *, $p < 0.05$. Value was significantly increased from non-GDX control. #, $p < 0.05$, Value at day 36 was significantly reduced from day 18 of GDX.

(B) Administering the LH implant resulted in a reduction in Aβ40 levels within the frontal cortex. Two-way ANOVA and pairwise comparison analysis revealed that compared to placebo treated animals, Aβ40 levels were significantly reduced following 36 days of treatment. Data is represented mean± SEM. For placebo day 18, $n = 4$, for placebo day 36 $n = 8$, for LH day 18 and 36 $n = 5$ /time point, *, $p < 0.05$. Value is significantly reduced from 36 day placebo treatment.

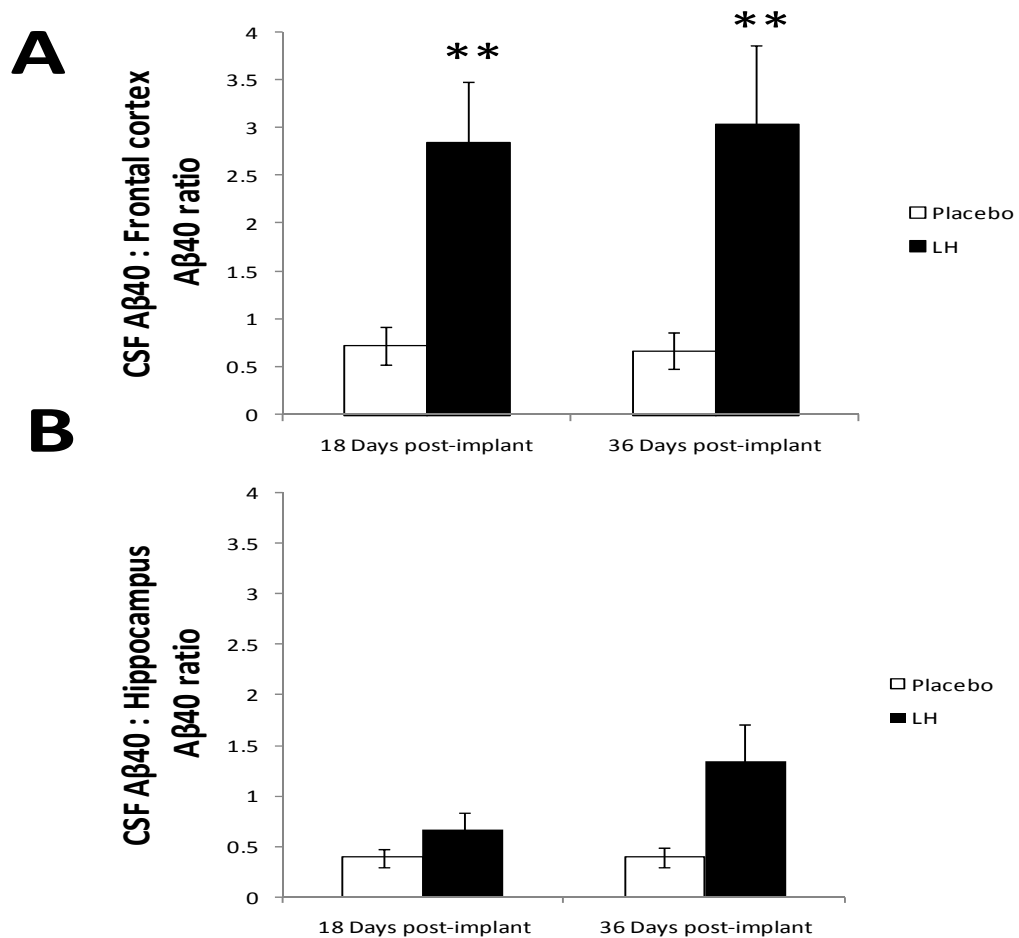
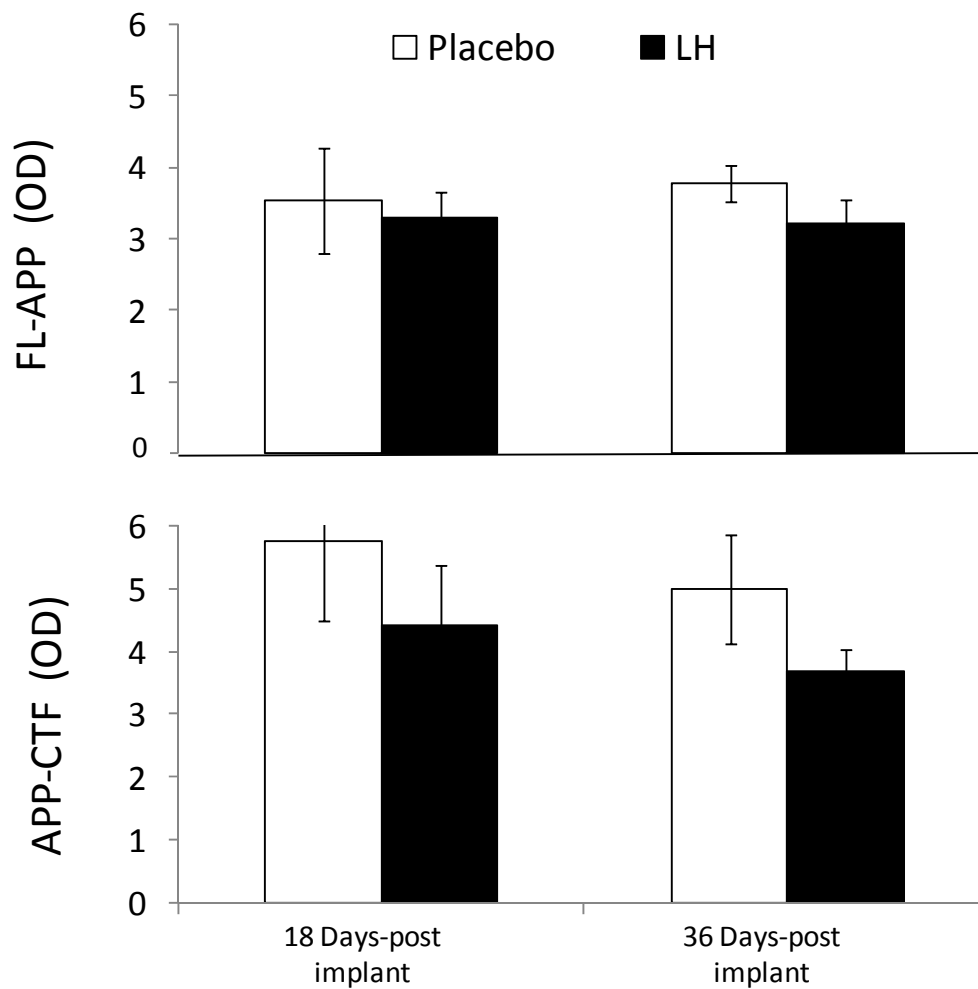
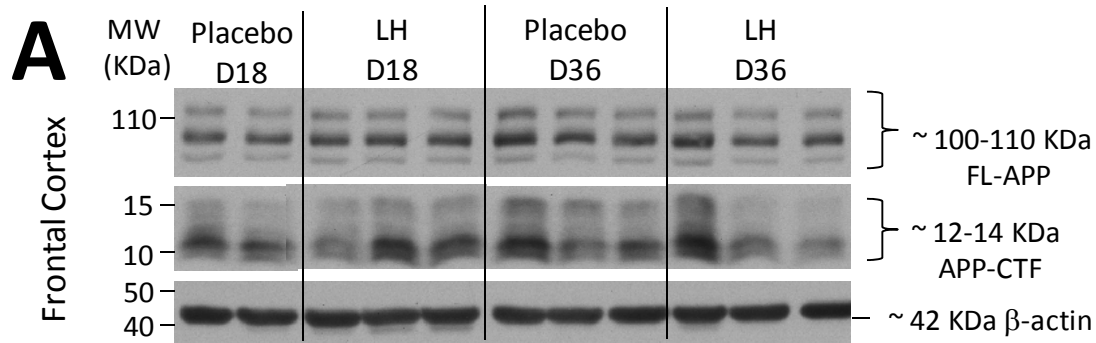


Figure 5.5 : Ratio of Aβ40 levels in the CSF to those in the frontal cortex (A) and hippocampus (B) of GDX guinea pigs administered placebo or LH for 18 or 36 days.(A) In the frontal cortex, there was main effect of treatment ($F=26.311$, $p<0.0001$). No effects of duration of treatment ($F=0.029$, $p<0.867$) and interaction effect between treatment and duration of treatment ($F=0.08$, $p<0.781$) impacted on rates of Aβ40 clearance. Compared to placebo, LH treatment significantly increased CSF Aβ40 to frontal cortex Aβ40 in both 18 and 36 days post implant. ** $p<0.0001$ (B) In the hippocampus, treatment ($F=8.336$, $p<0.011$), duration of treatment ($F=1.786$, $p<0.2$) and interaction effect between treatment and duration of treatment ($F=1.715$, $p<0.209$) did not impact on hippocampal Aβ40 levels. LH treatment led to a trend towards increased CSF Aβ40 to hippocampus Aβ40 ratio in both 18 and 36 days post-implant. Data is represented mean± SEM, for placebo day 18, $n=4$, for placebo day 36 $n=7$, for LH day 18 $n=5$ and LH day 36 $n=4$ pergroup.



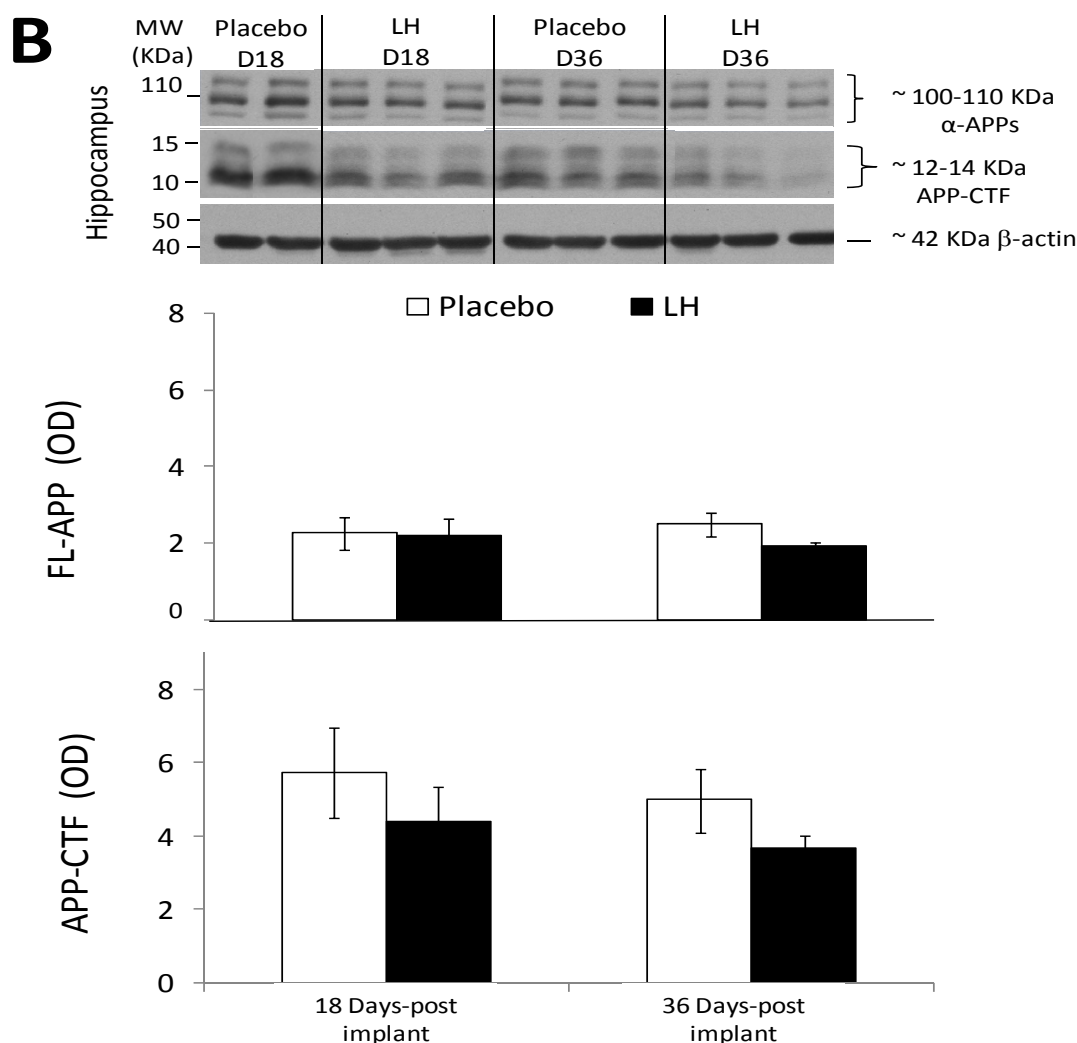


Figure 5.6 : Levels of full length APP and its C-terminal fragment in frontal cortex and hippocampus of GDX guinea pigs administered placebo or LH for 18 or 36 days.

Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 18 or 36 days and levels of APP and its C-terminal fragment (APP-CTF) were assessed by immunoblotting from tissue homogenates of (A) frontal cortex or (B) hippocampus. No significant changes were observed for FL-APP and its C-terminal fragment in both the frontal cortex and hippocampus. **Note** that the data from APP-CTF in the frontal cortex was not heterogeneous, thus statistical analysis was performed using the non parametric Kruskal Wallis analysis of variance. Data is represented as mean \pm SEM of 6 animals/ group.

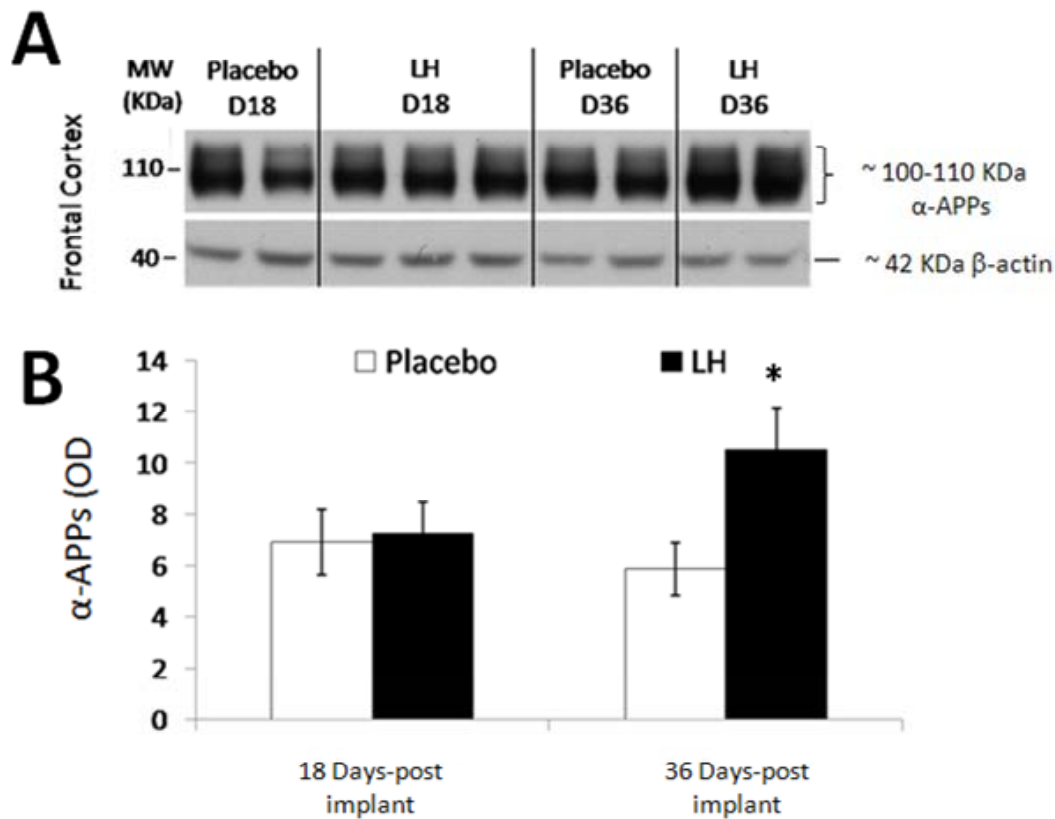


Figure 5.7: Levels of α -APPs in frontal cortex of GDX guinea pigs administered placebo or LH for 18 or 36 days.

Tissue homogenates from the frontal cortex underwent processing to isolate the soluble fraction to measure secreted α -APP by western immunoblotting(A). Administration of LH in the periphery increased the levels of α -APPs significantly at day 36 compared to the placebo groups(B). Two-way ANOVA and pairwise *post-hoc* analysis revealed that compared to placebo treated animals, α -APPs levels were significantly reduced following 36 days of treatment * $p < 0.05$, Value is significantly reduced from 36 day placebo treatment. Data is represented as mean \pm SEM of 6 animals/ group.

5.4.2 Cerebral A β levels are significantly reduced in leuprolide treated guinea pigs.

Previous studies in mice administered leuprolide have shown that this GnRH agonist reduced cerebral A β deposition (Bowen *et al.*, 2004; Casadesus *et al.*, 2006). The use of a larger rodent (non-transgenic) model such as the guinea pig allows regional differences in soluble brain A β levels and effects on CSF levels to be analysed.

GDX guinea pigs were administered a concentration of leuprolide for 18 or 36 days. Following treatment, animals were sacrificed and CSF and brain A β levels were analysed. The results are shown in Fig 5.7 and 5.8. Compared to animals administered placebo those treated with leuprolide did not show significantly altered CSF A β 40 levels. However a trend towards a reduction was observed following 18 days leuprolide treatment. Two-way ANOVA revealed there was a main effect of duration of treatment ($F=9.589$, $p<0.005$), rather than the treatment itself ($F=0.072$, $p<0.079$). This effect was particularly evident in the leuprolide group where there was a significant increase at day 36, compared to day 18 post-treatment ($p<0.012$).

Analysis of brain A β 40 levels revealed changes due to leuprolide treatment, particularly in the frontal cortex. Analysis of A β 40 levels in the frontal cortex revealed main effects of both treatment ($F=0.937$; $p<0.006$) and duration of treatment ($F=0.935$; $p<0.007$). Compared to placebo, leuprolide treatment initially led to a significant increase in A β 40 levels at 18 days ($p=0.031$), and then a reduction following 36 days of treatment ($p=0.024$) (Figure 5.8 A). However, analysis of hippocampal A β 40 levels showed no effects of the treatment ($F=1.31$, $p<0.266$) or duration of treatment ($F=0.09$, $p<0.768$) (Figure 5.8B).

Analysis of levels of APP and its C-terminal fragment in both the frontal cortex and hippocampus revealed no significant changes associated with leuprolide treatment (Figure 5.9). However, analysis of α -APPs levels showed that leuprolide increased the levels of this APP fragment. Two-way ANOVA revealed there were significant effects of leuprolide treatment ($F=28.158$, $p<0.001$) and duration of treatment ($F=9.51$, $p<0.006$). When compared to placebo there was a significant increase

following 18 ($p=0.002$) and 36 ($p=0.001$) days post-leuprolide treatment ($p<0.05$) (Figure 5.10).

Overall, the results show the most prominent effects of leuprolide were observed in the frontal cortex. The levels of A β 40 initially increased 18 days after treatment then reduced following 36 days of treatment. Prominent increases in α -APPs were also observed in the frontal cortex. No leuprolide mediated changes in A β 40 levels were observed in the CSF or hippocampus.

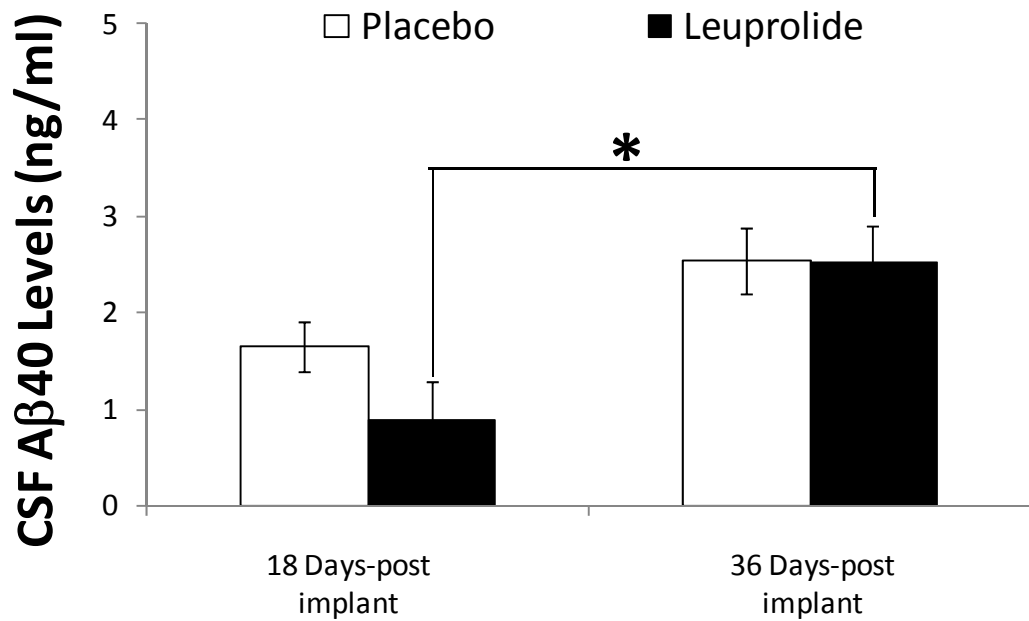


Figure 5.8 : CSF A β 40 levels from GDX guinea pigs administered placebo or leuprolide for 18 or 36 days. Gonadectomised (GDX) guinea pigs were administered placebo or leuprolide slow release pellet implants for 18 or 36 days and CSF was collected from the cisterna magna. Although not significant, at day 18 compared to animals administered placebo, those administered leuprolide showed reduced levels of CSFA β 40. Levels increased in both the placebo and leuprolide groups 36 days after administering the slow release implants. Data is represented as mean \pm SEM of 6 animals/group. Please note that CSFA β 40 from 2 samples of the day 18 leuprolidegroup,were not detectable by the ELISA assay.*, $p<0.05$, Values at day 36 significantly increased compared with animals treated with leuprolide for 18 days.

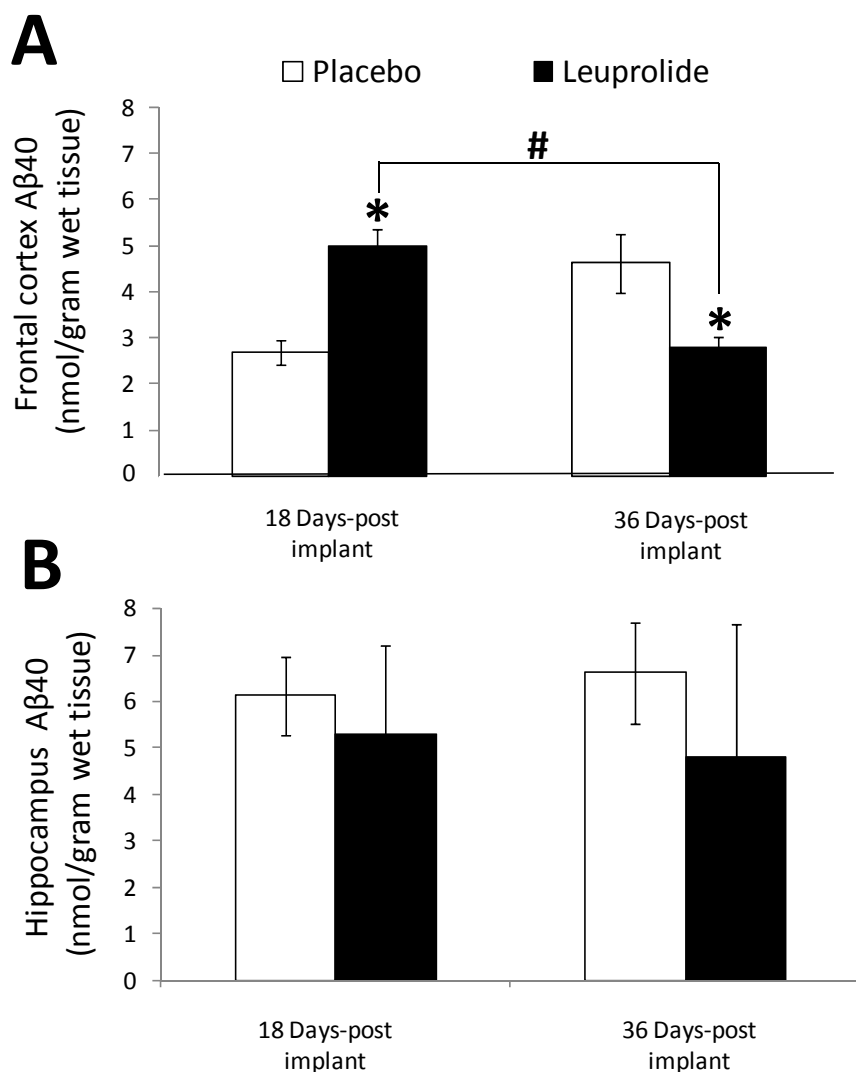


Figure 5.9 : A β 40 levels from frontal cortex and hippocampus of GDX guinea pigs administered placebo or leuprolide for 18 or 36 days. Gonadectomised (GDX) guinea pigs were administered placebo or leuprolide slow release pellet implants for 18 or 36 days and A β 40 levels were measured in the frontal cortex and hippocampus. (A) In the frontal cortex, treatment and duration of treatment impacted on A β 40 levels. Compared to placebo, leuprolide treatment initially led to a significant increase in A β 40 levels at 18 days, and then a reduction following 36 days of treatment. (B) In contrast to A β 40 levels in the frontal cortex, treatment or duration of treatment did not impact on hippocampal A β 40 levels. Data is represented as mean \pm SEM of 6 animals per group * p <0.05. Values significantly increased (Day18) or decreased (Day 36) in leuprolide treated animals. # p <0.05. Values at day 36 were significantly reduced compared with leuprolide treatment at day 18.

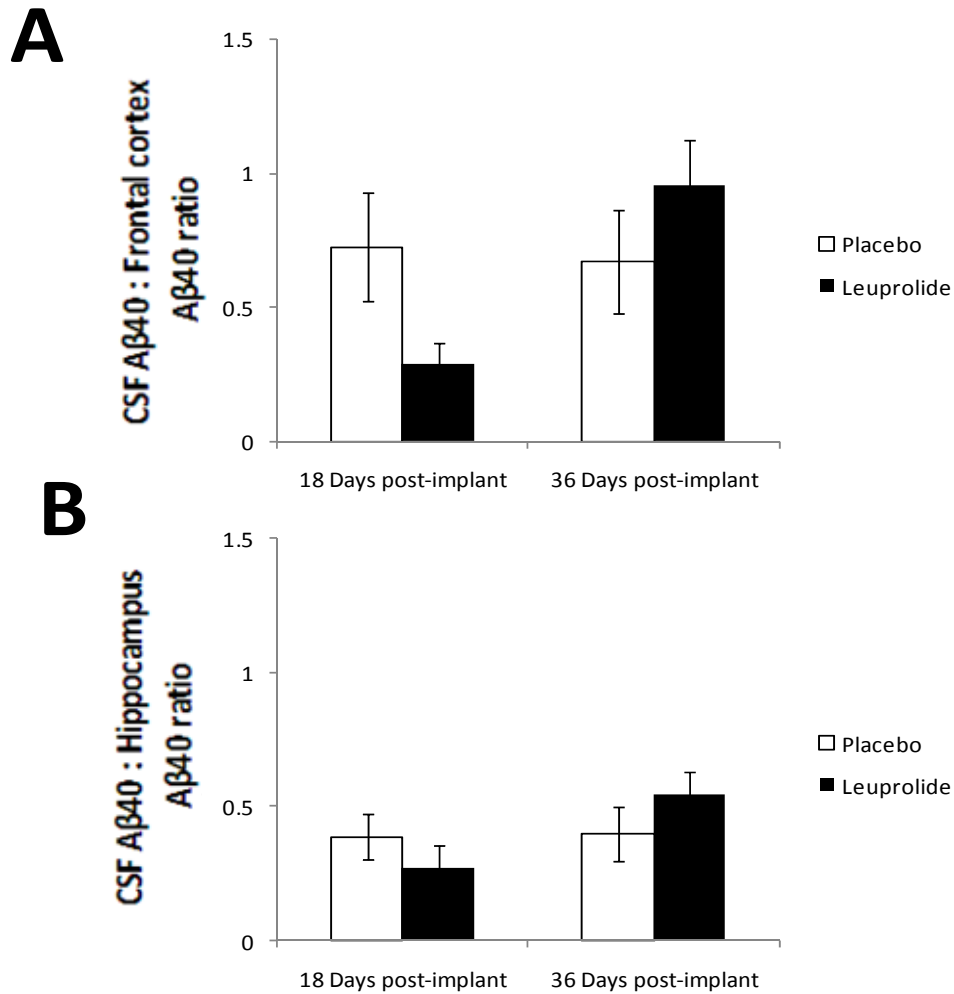
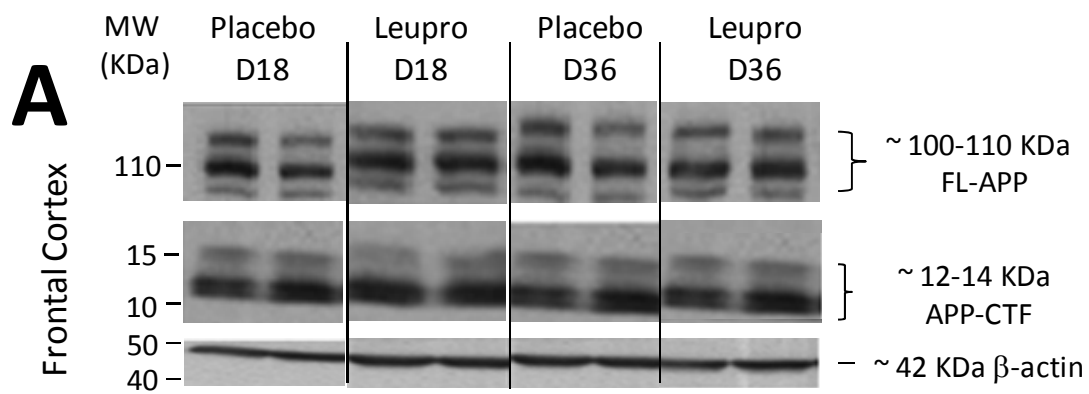
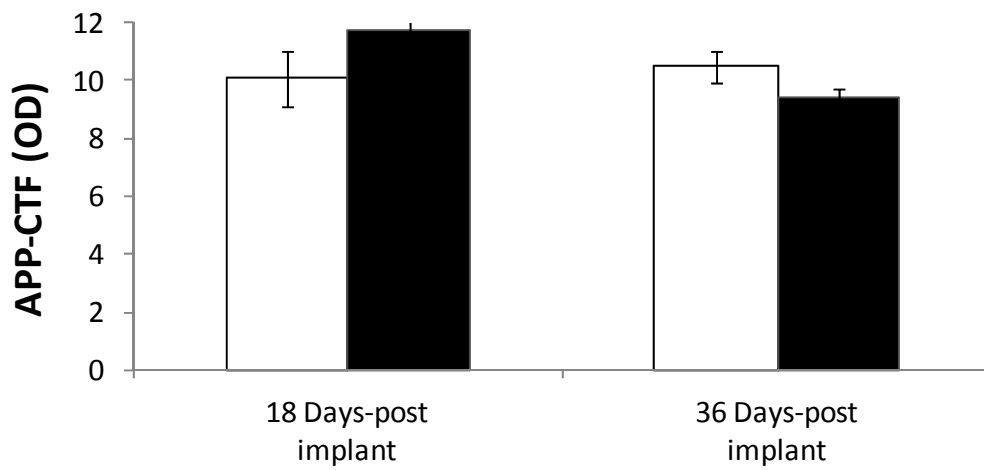
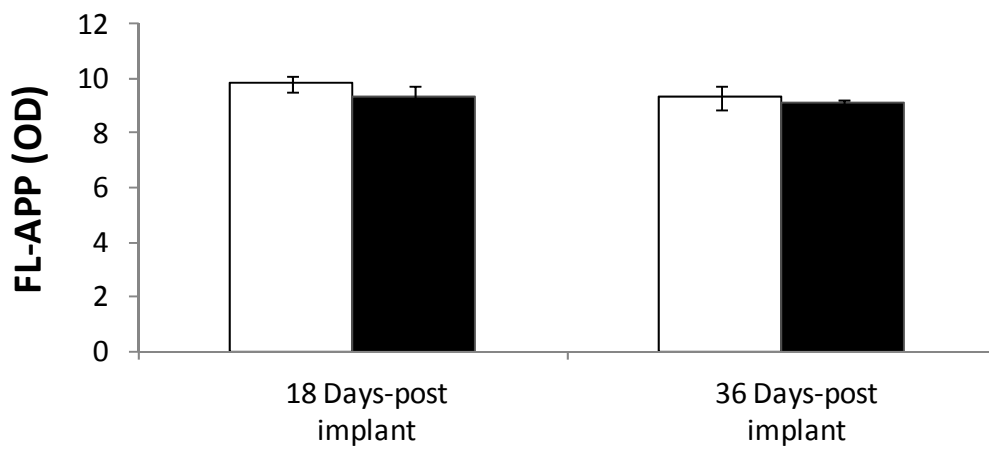


Figure 5.10 : Ratio of Aβ40 levels in the CSF to those in the frontal cortex (A) and hippocampus (B) of GDX guinea pigs administered placebo or leuprolide for 18 or 36 days. A ratio of Aβ40 levels in the CSF to those in (A) frontal cortex and (B) hippocampus was calculated and is presented as a bar graph. (A) In the frontal cortex, there were no treatment ($F=0.169$, $p<0.686$), duration of treatment ($F=2.577$, $p<0.127$) and interaction effect between treatment and duration of treatment ($F=3.515$, $p<0.078$) impacted on rates of Aβ40 clearance. Compared to placebo, leuprolide treatment led to a trend towards increases of CSF to Aβ40 ratio following 36 days of treatment. (B) In hippocampus, treatment ($F=0.018$, $p<0.894$), duration of treatment ($F=2.2$, $p<0.156$) and interaction effect between treatment and duration of treatment ($F=1.946$, $p<0.181$) did not impact on hippocampal Aβ40 levels. Similar trends was observed like in frontal cortex. Data is represented mean± SEM, for placebo day 18, $n=4$, for placebo day 36 $n=8$, for leuprolide day 18 $n=4$ and leuprolide day 36 $n=6$ /group.



□ Placebo ■ Leuprolide



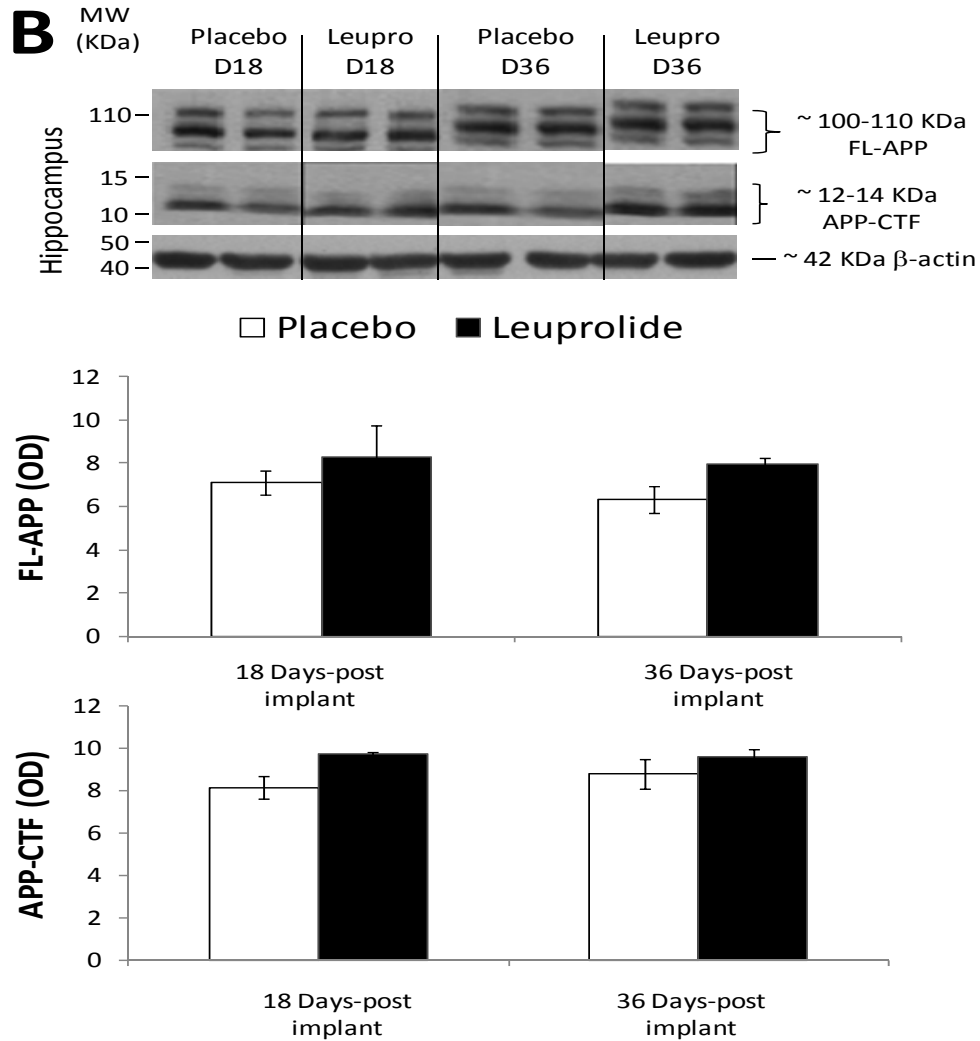


Figure 5.11 : Levels of full length APP and its C-terminal fragment in frontal cortex and hippocampus of GDX guinea pigs administered placebo or leuprolide for 18 or 36 days.

Gonadectomised (GDX) guinea pigs were administered placebo or LH slow release pellet implants for 18 or 36 days and levels of APP and its C-terminal fragment (APP-CTF) were assessed by immunoblotting from tissue homogenates of (A) frontal cortex or (B) hippocampus. (A) Treatment or duration of treatment did not significantly impact on FL-APP ($F=2.619, p<0.121$; $F=0.981, p<0.334$) or APP-CTF ($F=0.133, p<0.72$; $F=1.565, p<0.225$) in the frontal cortex. (B) Treatment or duration of treatment did not significantly impact on FL-APP ($F=2.509, p<0.129$; $F=0.416, p<0.526$) or APP-CTF ($F=6.191, p<0.22$; $F=0.327, p<0.574$) in the hippocampus. Data is represented as mean \pm SEM of 6 animals/ group.

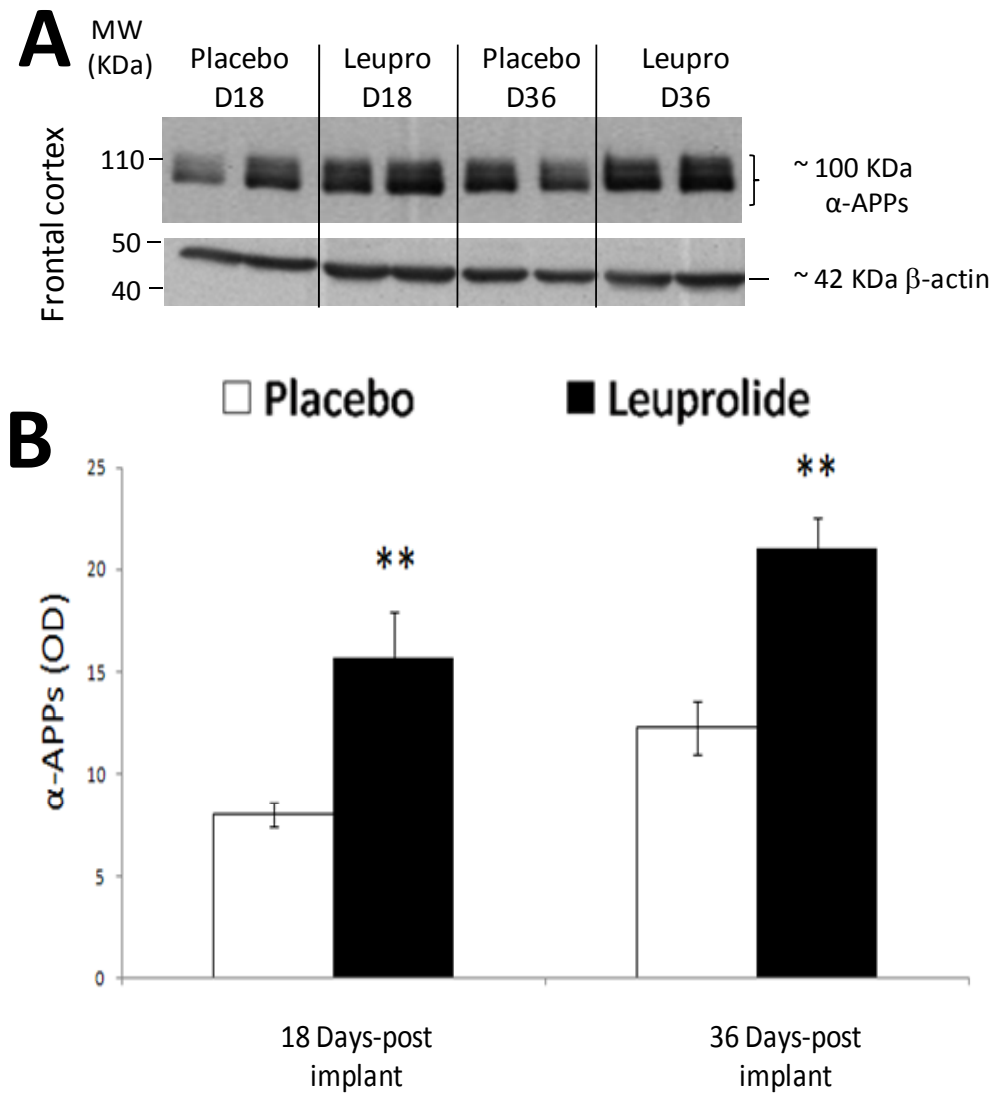


Figure 5.12: Levels of α -APPs in frontal cortex of GDX guinea pigs administered placebo or leuprolide for 18 or 36 days

(A) Homogenates from the frontal cortex underwent western immunoblotting for α -APPs using antibody WO2. Immunoblots show 2 representative samples from each group.

(B) Quantitative analysis of all samples followed by Two-way ANOVA analysis revealed a main effect of treatment ($F=28.158, p<0.001$) and duration of treatment ($F=9.51, p<0.006$). ** $p<0.01$ Values significantly increased compared to placebo. Data is represented as mean \pm SEM of 6 animals/ group.

5.4 Discussion

The findings from Chapter 4 provided initial evidence that direct exposure of the CNS to LH can alter cerebral A β metabolism. Whether this occurs under more physiological conditions is addressed in the current chapter where peripheral LH is administered as a slow release implant (Bowen *et al.*, 2004; Casadesus *et al.*, 2006). In addition, administration of the GnRH agonist (leuprolide acetate) was also investigated as it has been shown to reduce LH levels (Bowen *et al.*, 2004).

In Chapters 3 and 4, GDX was shown to increase CSF A β 40 levels, and the addition of LH was shown promote a further increase (Chapter 4, Fig 4.1), which was associated with increases in the frontal cortex and hippocampus. In contrast to these results, findings in the current chapter showed that peripheral addition of LH resulted in a time dependent increase in CSF A β 40 levels but a reduction in cerebral A β 40 levels. This reduction was more prominent in the frontal cortex where significant changes were observed earlier (18 days). However, by 36 days post- LH implantation, significant reductions were observed in both frontal cortex and hippocampus. The reduction in cerebral A β 40 levels associated with a corresponding increase of CSF A β 40 suggests that A β clearance is enhanced in guinea pigs administered LH. However changes were also observed in other APP metabolites, implying effects on APP processing. Although not significant a trend towards a reduction in full length APP and APP-CTF level was observed. This observation may account partially for the reduction in A β 40 levels within the frontal cortex and hippocampus, particularly at day 36 post-implant. However significant increases in α -APPs in post-ultracentrifugation supernatants of the frontal cortex were also observed.

To date current findings indicate that gonadotropins promote increased neuronal A β levels (Bowen *et al.*, 2004, Barron *et al.*, 2010). Furthermore, increased serum gonadotropins have been shown to be negatively associated with cognitive functioning in human (Rodrigues *et al.*, 2008, Hyde *et al.*, 2010) and animal studies (Casadesus *et al.*, 2007, Berry *et al.*, 2008, Barron *et al.*, 2010). Although peripheral LH administration did result in an increase in CSF A β 40 levels, surprisingly cerebral A β 40 levels were reduced. Of particular note is the contrasting results presented here

compared to that presented in chapter 4 (Wahjoepromono *et al.*, 2011), where the application of LH directly to the brain resulted in an increase in frontal cortex, hippocampal and CSF A β 40 levels. This is consistent with direct exposure of LH to neuronal cells *in vitro*, which has been shown to increase secreted and intracellular levels of A β , implicating a role for LH in promoting amyloidogenic processing of APP (Bowen *et al.*, 2004). The results presented in this current chapter however provide an argument against this earlier notion of LH action, as although treatment with LH increased CSF A β 40 levels, there is a marked reduction in cerebral A β levels.

The dose of LH used in the two experiments namely in Chapter 4 and this current Chapter were the same [2.25 ug/Kg (11.25 IU/Kg) BW/day]. Taken into account the body weight and blood volume of the guinea pigs used (500g and 35 ml, respectively), this dose is equivalent to 160 mIU/ml, which is about 16x and 5x the doses shown to increase the intracellular accumulation of A β and APP-CTFs and increase secretion of A β (10 mIU/ml and 30 mIU/ml, respectively) *in vitro*. The most likely explanation for the contrasting results is the site of LH application, which most likely led to a different level of exposure between the brain and the periphery. The direct application of slow release implants to the brain would be expected to lead to much higher levels of LH as opposed to its administration in the periphery. It could be argued that the high exposure to LH in the guinea pig brain described in Chapter 4 is similar to the situation where neurons exposed to LH, *in vitro*, result in the accumulation of A β (Bowen *et al.*, 2004).

Although studies have shown the presence of gonadotropins in CSF (Heller *et al.*, 1968, Knowles, 1972), one study has shown that a relatively small percentage (<1%) of peripheral gonadotropins (hCG), can cross the blood brain barrier into the CSF and hippocampus (Lukacs *et al.*, 1995). Further, LH is rapidly metabolized in the plasma with its half-life reported to be about 69 minutes (Schalch *et al.*, 1968).

In the experiments outlined in this Chapter the relatively rapid metabolism of LH would be compensated with a constant, daily release of LH ensuring relatively constant levels over a long term period. The concentration of LH used in this current study was about 5x less than the dose of its more potent analogue, hCG (60IU/kg/day) which was shown to cause a modest increase (1.5x) in soluble A β 40 levels in mice (Barron *et al.*, 2010).

Given the difference in potency of LH relative to hCG and the dose used it could be argued that minimal or no effects (as compared to direct exposure to the CSF) would have been observed. Surprisingly the converse is true where marked (opposite) effects in the CNS was observed when LH is administered peripherally.

The increase in CSF A β 40, decrease in cerebral A β 40 and increase in soluble α APPs, implies the LH may be acting to reduce cerebral A β , by actually promoting release of this neurotoxic peptide into the CSF. In favour of this notion there is evidence in the literature that supports increased secretion of A β and enhanced α APPs generation as a mechanism by which to reduce intracellular A β . Querfurth and Selkoe, (1994) showed that treatment with a calcium ionophore (that leads to an increase in intracellular calcium) increased cellular secretion of A β , APPs, and also the non-amyloidogenic fragment, p3. More recently, insulin has been shown to increase the secretion of α -APPs and A β , and reduce intracellular A β levels, a response that was mediated by MAPK signalling (Gasparini *et al.*, 2001). Similar findings were observed with latrepirdine (previously known as DimebonTM), an anti-histamine showing cognitive enhancing capabilities. Treatment of neuronal cells with latrepirdine led to an increase secretion of A β and α -APPs (Steele *et al.*, 2009). The latter study also showed that latrepirdine administered to transgenic mice promoted the release of A β into the interstitial fluid (ISF) and from isolated cortical synaptoneurosome. It was suggested that latrepirdine may enhance synaptic activity. Synaptic activity has been shown to modulate A β release into the ISF (Cirrito *et al.*, 2008) and promote the transport of APP to synapses and secretion of A β at the synapses, thereby reducing intraneuronal levels of A β (Tampellini *et al.*, 2009).

The studies above suggest, intracellular calcium levels and APP trafficking/ synaptic activity could all modulate the release of non-amyloidogenic and amyloidogenic fragments. It could be speculated that LH may activate these pathways thereby promoting release of APP metabolites and reduction in intracellular A β , however there is no strong evidence in the current findings presented in this chapter or in the literature that support this notion. Interestingly though, recent evidence indicates that intracellular Ca⁺² levels rise in bovine cumulus oocyte complexes (Silvestre *et al.*, 2012) and mouse leydig cells (Costa *et al.*, 2011) following exposure to increased LH. The study by Costa

et al (2011) further showed that the LH mediated increase in Ca^{+2} levels was attenuated by protein kinase (PKC and PKA) inhibitors, indicating that the LH mediated changes in intracellular calcium homeostasis is through activation of kinases (Costa *et al.*, 2011), which are known to regulate APP metabolism (Caporaso *et al.*, 1992; Buxbaum *et al.*, 1993; Gillespie *et al.*, 1992; Demaerschack *et al.*, 1993; Gabuzda *et al.*, 1993; Slack *et al.*, 1993; Hung *et al.*, 1993). Whether LH has a similar action in regulating calcium levels in neurons and whether this is mediated through activating protein kinases that regulate APP processing remains to be determined.

This Chapter also investigated whether, leuprolide acetate reduced cerebral $\text{A}\beta$ levels in guinea pigs similar to studies that have been reported in mice, (Bowen *et al.*, 2004, Casadesus *et al.*, 2006). In the Bowen *et al.*, study, a slow release leuprolide depot (1.5 mg/kg/BW) was injected monthly to C57Bl/6J mice for 8 weeks, resulting in a 1.5 fold and ~ 4 fold reduction in soluble cerebral $\text{A}\beta$ levels. Although serum gonadotropin levels were not measured, the action of leuprolide in reducing gonadotropin secretion indicated that it resulted in the reduction in cerebral $\text{A}\beta$ levels. In a more recognized model of AD, a slow release (depot) leuprolide formulation (7.5 mg/kg), was administered intramuscularly, twice monthly, for a period of 3 months to 21 months in APPswe mice. In this latter study although leuprolide treatment led to a ~ 4 fold reduction in serum LH levels, there was a significant but modest $\sim 20\%$ reduction in amyloid deposition (Casadesus *et al.*, 2006).

The results presented in this Chapter extended the previous findings by showing that leuprolide modulated cerebral $\text{A}\beta$ levels in a time dependent manner, which lead to contrasting trends in CSF $\text{A}\beta_{40}$ levels compared to cerebral $\text{A}\beta_{40}$ levels. Here, administering leuprolide led to an initial increase at day 18 post-treatment. Following 36 days post-treatment a significant reduction compared to placebo, was observed. This effect was most prominent in the frontal cortex when compared to the hippocampus. In contrast, the ratio of CSF $\text{A}\beta_{40}$ to frontal cortex levels was reduced following 18 days of treatment, and then increased by 36 days of treatment. These findings parallel the effects of leuprolide on altering gonadotropin levels where it results in an initial increase or “flare effects” in serum gonadotropin secretion (generally lasting 10-20

days), followed by a precipitous reduction (Okada *et al.*, 1996, Weckermann *et al.*, 2004).

The experiments outlined in this current Chapter used a lower concentration of leuprolide than that used in the Bowen *et al* (2004) and Casadesus *et al* (2006) studies. In addition, leuprolide was administered via a slow release implant, mimicking chronic reductions in gonadotropins. Serum LH levels were not measured in this study due to difficulties in detecting guinea pig LH (see Chapter 4). However, similar studies have shown that administering leuprolide via slow release implants lead to marked reductions in serum LH following initial spikes (Okada *et al.*, 1996).

A very recent study also administered leuprolide to the 3xTg AD mouse model via slow release implants, but showed contrasting effects to that observed in mice or our guinea pig model. In this study, Rosario and colleagues (2012) administered leuprolide to sham operated male mice and showed it did not lower cerebral amyloid deposition or improve hippocampal dependent memory (compared to GDX animals only). Further, when GDX mice were administered leuprolide in combination with testosterone, it negated the benefits of administering testosterone only at reducing cerebral amyloid load and improving memory. It is difficult to explain the contrasting results to previous rodent studies. Highlighted by Rosario and colleagues was the differences in sex where the Bowen and Casadesus studies used female mice. Evidence exists indicating significant sex differences in severity of pathology in AD mouse models (Carroll *et al.*, 2010a; Oliveira *et al.*, 2011).and leuprolide has been shown to have benefits at improving hippocampal dependent memory in female rat models (Bryan *et al.*, 2010, (Ziegler and Thornton, 2010). Together, these studies give precedence to the possible sex-specific benefits which could be further addressed by a direct comparison of the effects of leuprolide on the brains of OVX female and GDX male mice.

It is noteworthy that the two transgenic mouse studies (Casadesus *et al.*, 2006, Rosario *et al.*, 2012), showed a subtle or no reduction in amyloid deposition (assessed by immunohistochemistry), whilst, similar to the current study, the Bowen *et al.*, 2004, showed >2 fold reduction in soluble A β levels (assessed by ELISA) in the brains of wild-type mice. Transgenic mice models exhibit a marked over-expression of cerebral

human APP and A β , which may mask the potency of leuprolide at modulating A β levels or the deposition of amyloid. As such it can not completely rule out the possibility that leuprolide can still modulate the metabolism of endogenous A β resulting in reduced soluble A β levels.

Although plaque deposition is not evident in guinea pig brain, guinea pig A β has the capacity to form higher order molecular structures, such as oligomers (Beck, Bruckner *et al.* 2000), widely considered to be the principal contributor to neurotoxicity in AD pathogenesis. Thus a reduction in this species would be highly relevant to AD. Whether leuprolide may reduce guinea pig A β levels in the frontal cortex by modulating APP metabolism is not clear. In this Chapter, increases in α -APPs and corresponding reductions in A β 40 levels observed following 36 days leuprolide treatment (Fig 5.9A and 5.12 respectively) and no changes in CSFA β 40 levels (Fig 5.8) at this time point suggests that leuprolide may promote non-amyloidogenic processing of APP.

Overall the findings from this Chapter indicate that administering human LH to the guinea pig periphery can alter CSF and cerebral A β 40 levels, but unexpectedly, reduces cerebral and increases CSF A β 40 levels. This provides novel data that can be extended to further in *in vitro* experiments to provide insight into the mechanisms by which LH could impact on APP processing or A β clearance pathways. Administering the GnRH agonist, leuprolide to transgenic mouse models have shown no or subtle changes in cerebral A β 40 levels. The results presented in this Chapter have provided evidence in a non-transgenic animal model that leuprolide can significantly reduce cerebral A β 40 levels, perhaps providing a more suitable model for assessing the benefits of leuprolide on AD related proteins.

Conclusion

Chapters 3-5 have investigated the impact of key hormones of the HPG axis, testosterone and LH on CSF and cerebral A β levels in the guinea pig. Chapter 4 and 5 have provided evidence for a role of LH in modulating A β levels. Chapter 4 has shown that the direct application of human LH to the guinea pig CNS, results in an accumulation of A β in a similar manner that has been reported for neuronal cells in

culture and *in vivo*. In contrast, the application of human LH to the guinea pig periphery resulted in reduced cerebral A β levels. Although not consistent with findings obtained in mouse models, it provides an interesting line of research that can be further explored in the guinea pig.

The findings presented in Chapter 3 confirm the effects of testosterone depletion and supplementation in the guinea pig. Consistent with other *in vivo* models, testosterone depletion through GDX led to an increase in CNS A β levels, while testosterone supplementation prevented this increase. The following chapters assess the benefits of testosterone supplementation on cognition, blood and imaging biomarkers in a cohort of men with testosterone levels in the lower part of the normal range.

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Chapter 6

Effect of testosterone supplementation on plasma biomarkers in men with subjective memory complaints (SMC)

6.1 Introduction

Male aging is accompanied by a gradual decline in testosterone activity (andropause). This can manifest itself in many ways, including sexual dysfunction, diminished physical stamina, loss of muscle and bone mass, systolic hypertension, the development of abdominal visceral fat mass, insulin resistance, high levels of cholesterol, LDL and triglycerides, low HDL levels, and impaired quality of life with symptoms such as depression and cognitive impairment (Kenny *et al.*, 2001; Moffat *et al.*, 2002; Schroeder *et al.*, 2004; Wang *et al.*, 1996). The decline in testosterone levels starts in the fifth decade of life (Gray *et al.*, 1991), with men 70 years of age and older having the lowest levels. Estimates for the prevalence of men with low levels of testosterone range from 1 in 200 (Morley, J.E, 2000), 1 in 20 (Araujo *et al.*, 2007) to 1 in 4 (Leifke 2000), depending on age of population, ethnicity and health factors. Such men are candidates for carefully monitored testosterone replacement therapy.

Many studies have already been undertaken regarding testosterone replacement therapy. However, there is a lack of data based on longitudinal, double-blind, randomized placebo-controlled clinical studies of aging men which have investigated comprehensively testosterone's long-term effects on a wide range of aspects of men's health including the cardiovascular system, the immune system, body metabolism, and the brain. Although men in their sixties (in general) are less prone to AD than women of the same age (Jorm, 1987), evidence suggests that testosterone deficiency is associated with a higher risk for AD (Hogervorst *et al.*, 2004; Rosario *et al.*, 2004). Importantly, a study by Moffat *et al.* 2004 indicates that the deficiency precedes the onset of dementia.

In the previous chapter, castrated guinea pigs were used as animal models to mimic the condition of testosterone deficiency in men. Testosterone treatment in these animals was shown to reduce CSF and cerebral A β levels. In cell culture studies of primary rat cortical neurons testosterone has also been shown to decrease A β levels (Gouras *et al.*, 2000). These findings suggest that testosterone supplementation in elderly men may protect against AD. Clinical studies do in fact suggest that testosterone levels can influence A β levels. For example, Gandy *et al.* 2001 recorded an elevation in plasma A β

levels in men being treated with testosterone suppression drugs for prostate cancer. In addition, Almeida *et al.* 2004 found that low testosterone levels are associated with increased plasma A β levels in men. Testosterone supplementation in men who have AD or mild cognitive impairment (MCI) has been shown to improve spatial memory and cognition (Tan *et al.*, 2003; Cherrier *et al.*, 2005). Although there have been some inconsistencies in study results, most studies suggest that testosterone replacement therapy might prevent or delay AD in testosterone deficient men (Traish *et al.*, 2011).

Almost universally, a testosterone deficient state in older men is permanent and as a result, testosterone replacement therapy (TRT) may be for life. Therefore, any testosterone treatment would have to be very carefully designed and well researched. The best approach in a clinical context would be via double-blind, randomized, cross-over, placebo-controlled trials. Improvements in healthcare over the last few decades have increased life expectancy considerably. Therefore the increased risks associated with age-related low levels of testosterone may have massive implications for both the individuals concerned and the community faced with the prospect of an aging population that requires more health care facilities than we currently provide.

For many decades it was thought that raising testosterone levels would increase the risk of prostate cancer. Over the last 10 years however, there has been much debate about this theory, the evidence for this has been disputed, and in fact testosterone is now believed not to increase the risk, even in people already at high risk of developing prostate cancer (Rinnab *et al.*, 2009 ;Feneley and Carruthers 2012 in press).

Some studies have linked testosterone treatment to other important adverse health outcomes such as increased risks of atherosclerosis and depressive illness. However, studies showing unfavourable outcomes usually concern people deliberately trying to achieve supraphysiological levels for the purpose of improving athletic performance, for example (Aaronson *et al.*, 2011). Results from such studies do not apply to patients trying to maintain testosterone within a normal physiological range. However, extra care needs to be taken in order to balance the possible therapeutic benefits against the potential risks. This chapter describes a clinical study that was undertaken to investigate the effect of testosterone treatment in a group of elderly with testosterone in the lower part of the normal range and below. Parameters that were investigated include blood and

brain imaging biomarkers and cognitive performance. Side-effects of the treatment were also monitored. The blood biomarkers that were measured include testosterone and its derivative dihydrotestosterone (DHT), SHBG, Calculated Free Testosterone, estradiol, luteinizing hormone (LH), insulin, PSA levels, and plasma A β levels. Lipid profiles of the participants were also characterised. The effect of testosterone on cognitive performance and brain imaging biomarkers will be presented in chapters 7 and chapter 8 respectively.

6.2 Aims

The aims of this study were :

- To investigate the effect of testosterone administration on plasma levels of testosterone, its derivative dihydrotestosterone (DHT), Calculated Free Testosterone, and its aromatization product estradiol.
- To investigate the effect of testosterone treatment on luteinizing hormone (LH) levels in blood plasma.
- To investigate the effect of testosterone administration on lipid profiles in blood plasma, which include levels of cholesterol, triglyceride, LDL, HDL, Chol/HDL and calculated LDL/ HDL ratios.
- To investigate whether testosterone treatment in men with low normal and below reduces plasma A β levels.
- To investigate the effects of testosterone treatment on plasma insulin levels.
- To investigate the effect of testosterone treatment on Prostate Specific Antigen (PSA) levels in blood plasma.
- To determine whether there is an APOE genotype specific response to testosterone treatment.

6.3 Methods

Participants

Forty-four of the 50 subjective memory complainers men who met the eligibility criteria completed the study. They were recruited and assigned to one of the two parallel groups; placebo (n=22) or testosterone (n=22) treatment. Assignment was random using stratification to ensure each arm of the study was balanced, in terms of individual memory performance at baseline. The study design was such that participants received testosterone treatment or placebo for a period of 24 weeks, followed by a washout period of 4 weeks, after which time, the participant groups were “crossed over”, such that the placebo group received testosterone and *vice versa* for a further 24 weeks. For all participants, there were a total of 11 clinic visits to Siloam Hospital, Lippo Karawaci (Indonesia), within the 52-week study period. The participants and the investigators were blinded to the treatment assignment until the completion of the study.

Procedure

During the first (baseline) visit, participant blood pressure, height, weight, body fat percentage and body mass index (BMI) were checked and blood was drawn. Blood samples (\pm 45 ml) were collected using serum separating tubes (SST, that include a gel and blood clot activator), EDTA, and heparin collection tubes. Blood in SST collection tubes were centrifuged at 2500 rpm for 10 min to separate the serum. This serum was removed, divided into 1 ml aliquots to which 10 μ l (100 μ g/ml) of protein inhibitor (aprotinin – Sigma) was added. Blood samples in EDTA and heparin collection tubes were centrifuged at 1000

rpm for 10 min at RT, to separate the plasma, leucocytes, and red blood cells. Plasma was removed and centrifuged further at 2500 rpm for 10 min to harvest platelets. The resulting supernatant (plasma) was aliquoted (~ 1 to 5 ml lots) and 10µl protein inhibitor (aprotinin – Sigma) was added to each ml of plasma. These blood samples were used for baseline readings of blood counts, serum testosterone levels, levels of LH and sex hormone binding globulin (SHBG), DHT (dihydrotestosterone), estradiol, cholesterol, insulin, glucose, PSA, plasma Aβ levels and lipid (as described in section 2.1.1.2.2). The participants' *APOE* genotypes were also determined.

For the testosterone treatment group (n=22), testosterone (50 mg in the form of Andromen[®] FORTE cream) was applied daily to the scrotum, thus using a transdermal route (topically), for a period of 24 weeks. For the placebo group (n=22) the same amount of the cream base [dl-α tocopherol acetate (vitamin E), without the active ingredient, testosterone] was applied with the same frequency. At each of the six remaining clinic visits i.e. at 4, 8, 12, 16, 20 and 24 weeks during the first treatment period, blood collection was carried out as described for the baseline measurement. During the 4 week washout period, the participants were given an equivalent amount of cream base lacking testosterone and vitamin E that was also applied to the scrotum. The placebo and testosterone treatment arms of the study were then crossed-over for a further 24-weeks treatment period. During this period, the participants were examined every eight weeks (three bleeds in total i.e. at weeks 8, 16 and 24 of the cross-over period); and blood samples were collected for analysis as described previously.

Blood biomarker measurement

Serum samples (n=535) were analyzed for total testosterone (TT) and dihydrotestosterone (DHT) using an isotope dilution LC-MS/MS method at the Anzac Research Institute, NSW (Handelsman *et al*, 2011). Other serum samples were analyzed for LH, estradiol, PSA, SHBG and insulin in Pathwest

Laboratory, Perth, Western Australia and plasma heparin were analyzed for lipid profile and albumin. Serum free testosterone (CFT) was calculated from TT, SHBG and albumin using the standard formula of Vermeulen, 1999.. Plasma A β was measured using an ELISA developed in Prof Martins laboratory (Mehta *et al.*, 2000).

Statistical analysis

Statistical analysis was performed using The Statistical Package of Social Sciences (SPSS version 19, SPSS Inc, Chicago, IL). The Kolmogorov-Smirnov and Levene's test was used to examine normality of distribution of continuous variables for each group. The analysis consisted of repeated-measures analysis of variance to compare between treatment groups, within-subject changes over the course of the treatment period. For parametric data, one way ANOVA repeated measures were performed to look at changes in blood biomarkers over time and following the different treatments, assuming no carry-over effects of testosterone treatment. Carry over effects were tested from differences at baseline in the first period (week 0) and at the washout period (week 24-28, which is baseline for the second period). For non-parametric data, Friedman's Test and/or Wilcoxon signed-rank test was performed. When directly comparing two groups from both arms of the study, as well as comparing two groups based on the presence of APOE ϵ 4, two tailed independent t-tests were performed. All analyses were two-tailed and the alpha level was set at .05.

6.4 Results

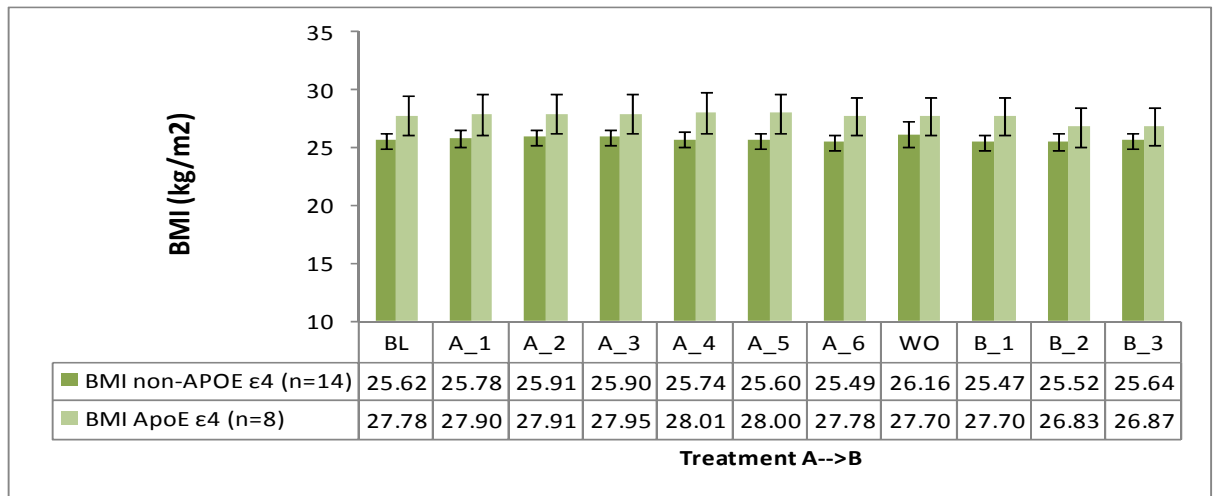
Participant characteristics at baseline

There were no differences between the two treatment groups of the study in terms of age, years of education, APOE allele status, and Body Mass Index (BMI) as shown in Table 6.1. There were also no significant differences observed in BMI and % body fat over time for both arms (Figure 6.1a and 6.1b).

Table 6.1 Mean (SD) age, education, Body Mass Index (BMI), and % Body Fat at baseline

Group	n	Age	Education	BMI	% Body Fat
Treatment A→B	22	59.22 (7.18)	13.95 (2.84)	26.4 (3.64)	24.29 (2.65)
APOE ε 4-	14	59 (5.82)	13.79 (3.31)	25.62 (2.6)	23 (1.16)
APOE ε4+	8	59.625 (9.58)	14.25 (1.91)	27.77 (4.9)	25.58 (2.51)
Treatment B-->A	22	62.91 (8.22)	13.76 (3.39)	24.25 (3.83)	22.05 (1.75)
APOE ε 4-	18	62.72 (7.31)	13.47 (3.62)	24.18 (3.92)	20.9 (1.58)
APOE ε4+	4	63.75 (13)	15 (2)	24.55 (3.91)	23.2 (1.98)

(A)



(B)

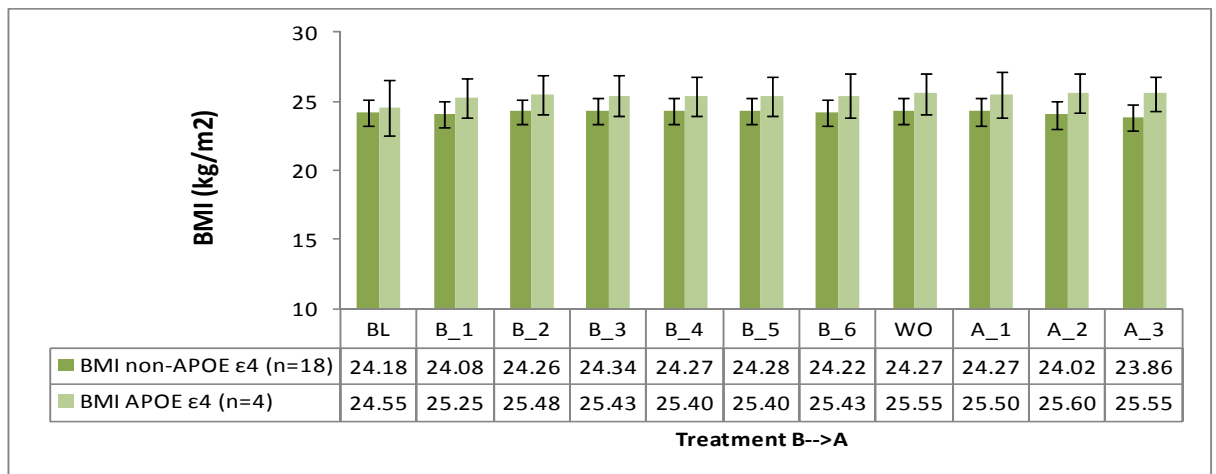
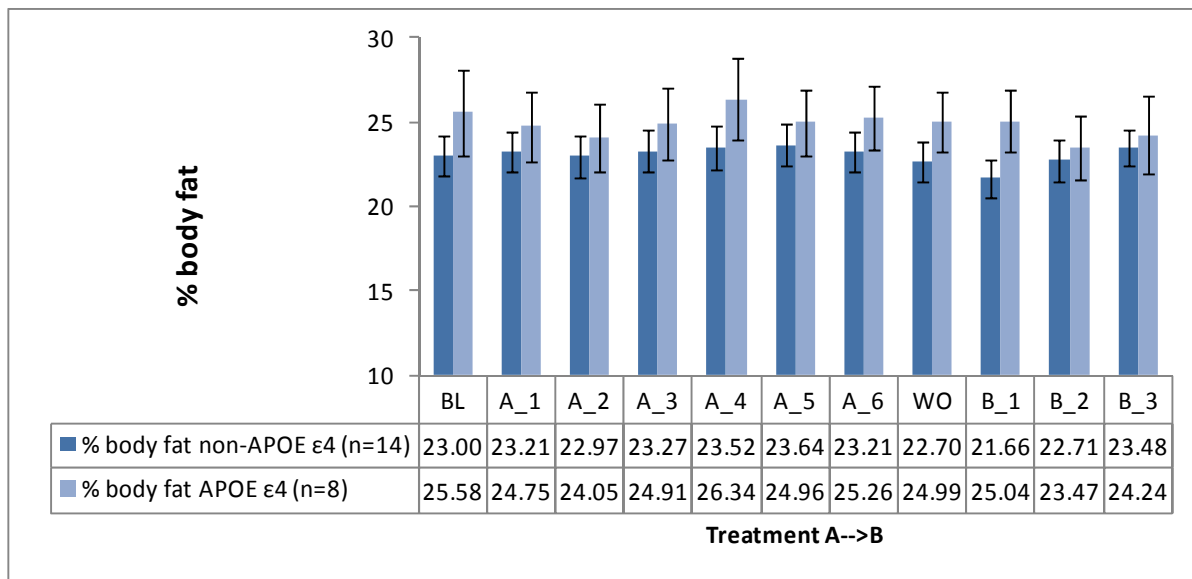


Figure 6.1(a) : Comparison of non-APOE ε4 and APOE ε4 carriers for Body Mass Index (BMI) (n=22) at 11 visits in treatment group A→B (A) and treatment group B→A (B). The bars represent the mean \pm standard deviation. Values in kg/m² underneath the bars are the mean of each group. No differences were observed between any of the groups of either arm, and APOEε4 status had no effect. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

(A)



(B)

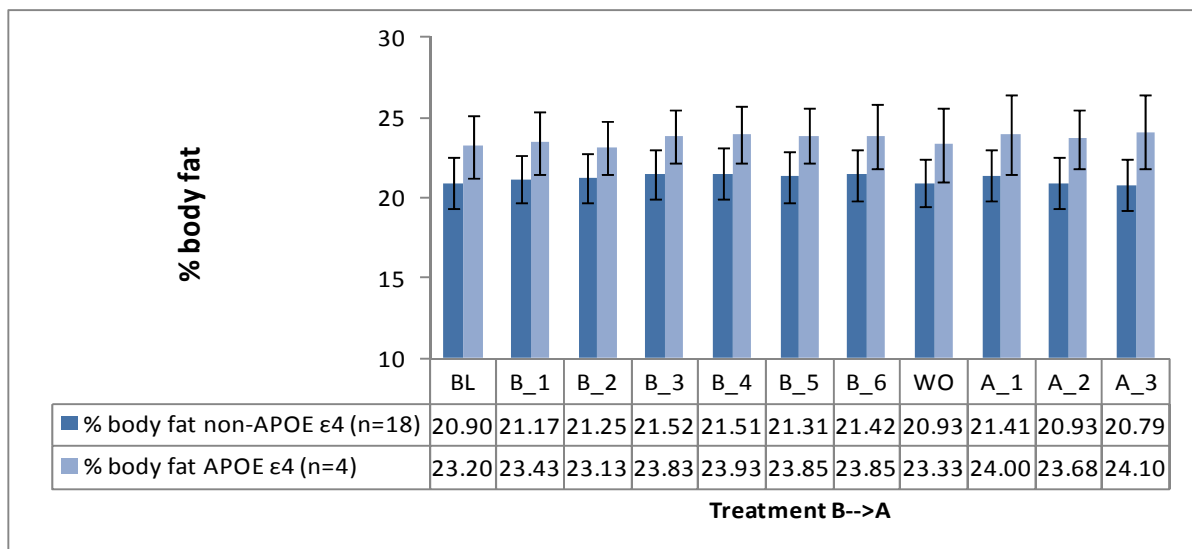


Figure 6.1(b) : Comparison of non-APOE ε4 and APOE ε4 carriers for % Body Fat (n=22) at 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in percentage underneath the bars are the mean of each group. No differences were observed between any of the groups of the two arms, and APOEε4 allele status did not influence results. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

6.4.1 Effects of testosterone administration on plasma total testosterone levels and its by-products, dihydrotestosterone (DHT) and estradiol.

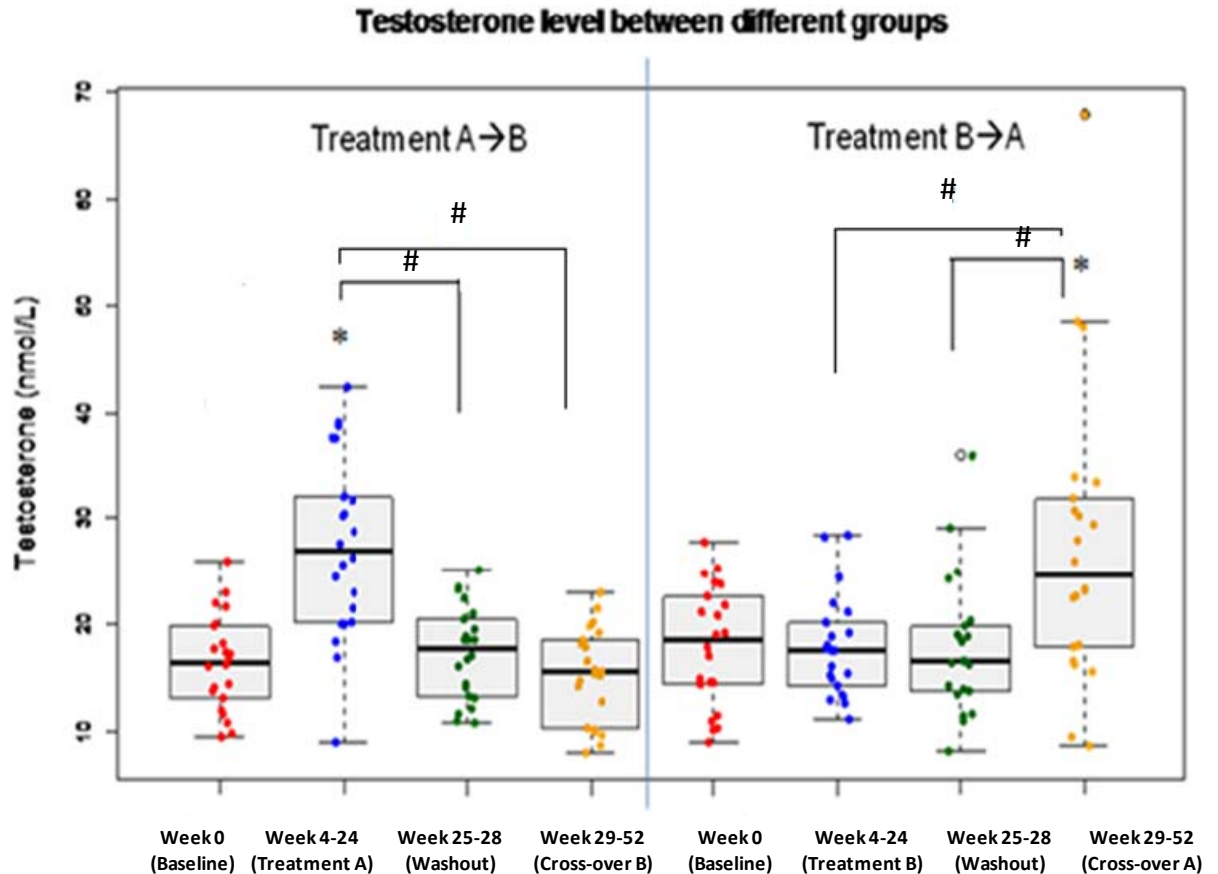
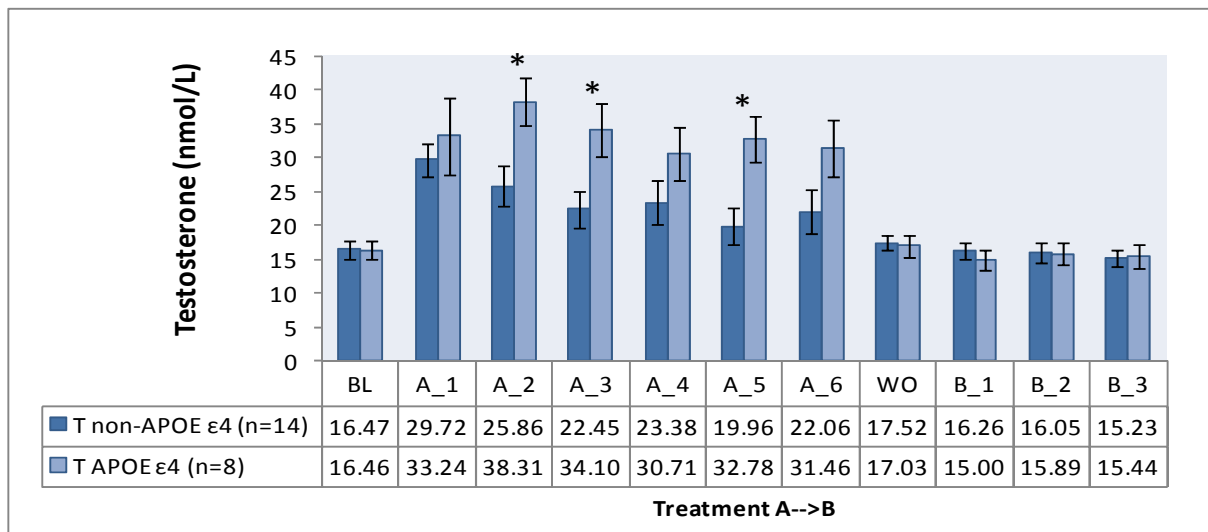


Figure 6.2 : Boxplots of total serum testosterone levels for both arms at 4 different time points: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data were in nmol/L and are presented as median \pm quartiles. Values in the first treatment are the averages of 6 measurements and values given for the second treatment period (Cross-over) are the average of 3 measurements. Testosterone levels were significantly increased in treatment A and cross-over A (ie testosterone treatment times) compared to all other treatment periods in the two arms. * $p < 0.05$ in comparison to baseline of each arm of the treatments, # $p < 0.05$ in comparison between time points (shown by brackets).

(A)



(B)

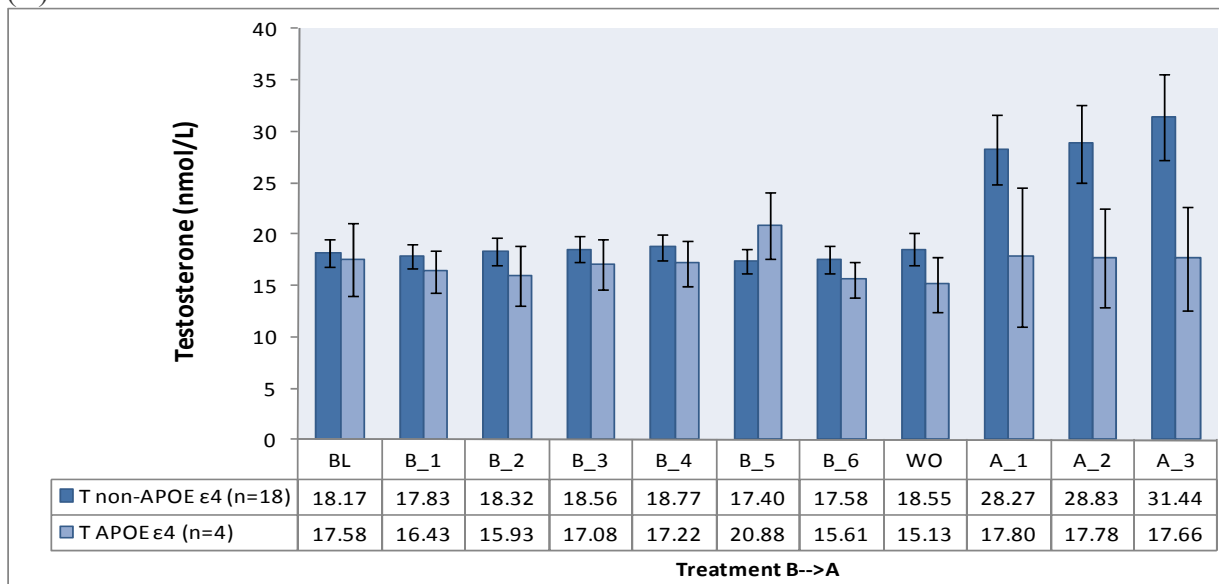


Figure 6.3 : Comparison of serum testosterone levels in APOE ε4 and non-APOE ε4 carriers (n=22) at 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in nmol/L underneath the bars are the mean of each group. Significant differences were observed between non-APOE ε4 and APOE ε4 group in treatment arm A→B (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

Dihydrotestosterone (DHT), a peripheral conversion product of testosterone, was also monitored (Figure 6.4). Free or unbound testosterone resulting from administration of transdermal testosterone will have entered the prostate and been reduced to some degree by 5-alpha reductase to DHT, which would then have been able to bind to androgen receptors.

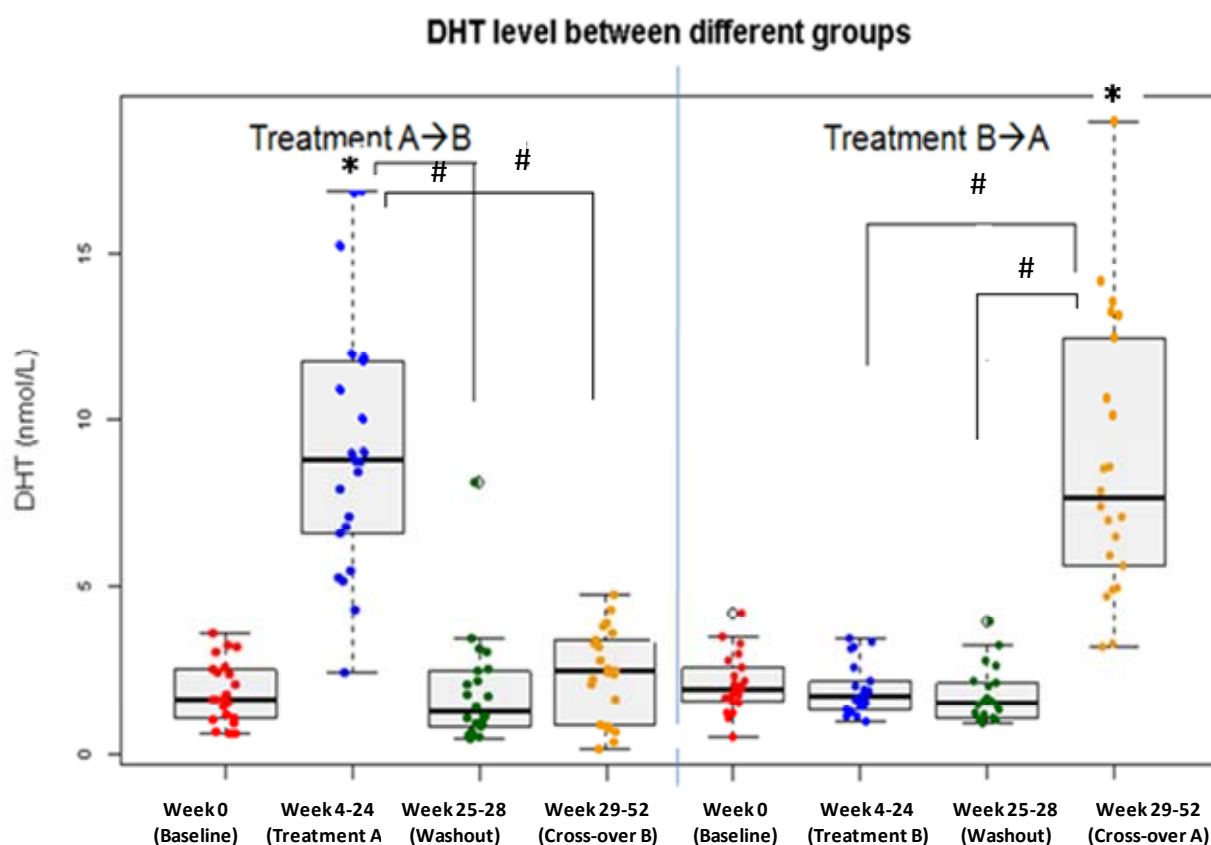
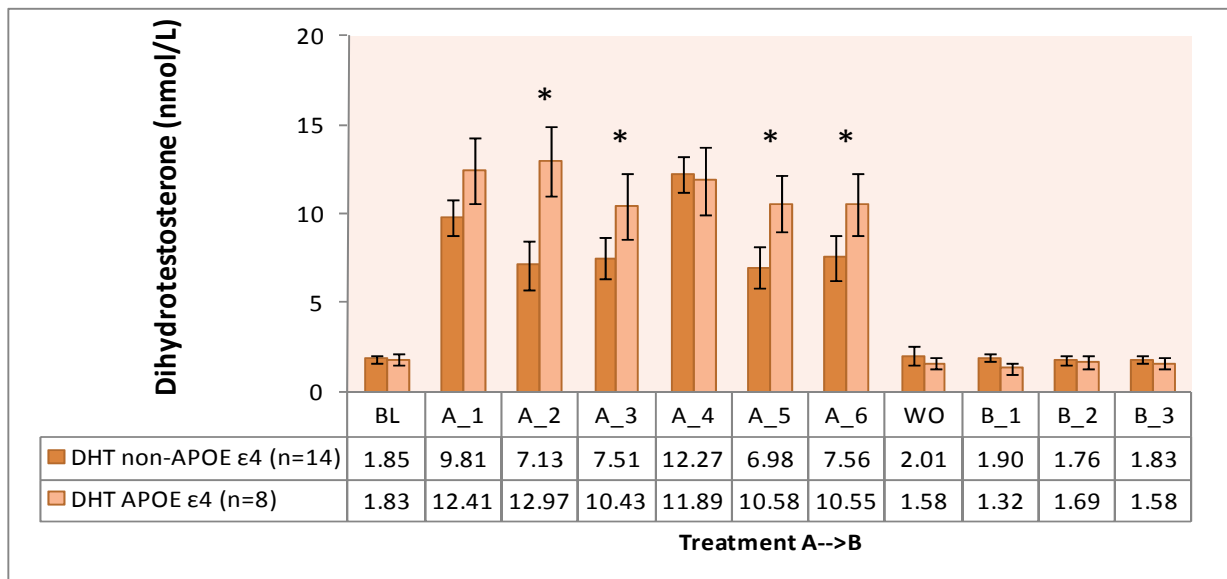


Figure 6.4 : Boxplots of serum dihydrotestosterone (DHT) levels for both arms at 4 different time points : Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in nmol/L and are presented as median \pm quartiles. Values shown in first treatment are the averages from 6 visits and values in the second treatment time (Cross-over) were averaged from 3 visits. Similar to total testosterone levels, DHT levels were significantly increased in treatment A and cross-over A compared to all groups in both arms. * $p < 0.05$ in comparisons to baseline of each arm of the treatments, # $p < 0.05$ in comparisons between groups designated by brackets.

(A)



(B)

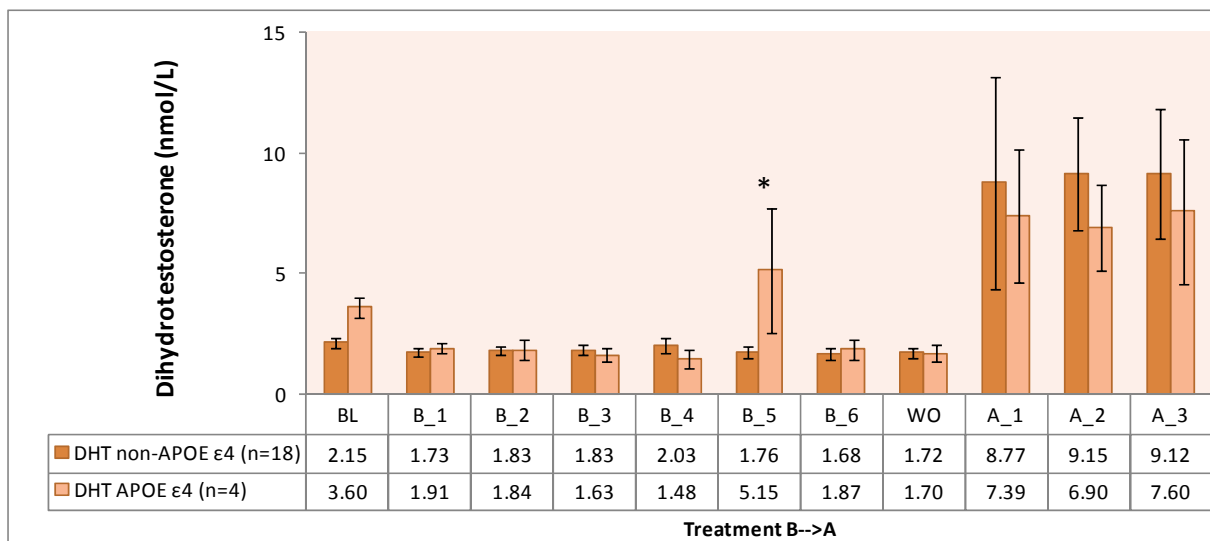


Figure 6.5 : Comparison of serum DHT levels in APOE ε4 and non-APOE ε4 carriers (n=22) at the individual 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in nmol/L underneath the bars are the mean of each group. Significant differences were observed between non-APOE ε4 and APOE ε4 group in both treatment arm (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

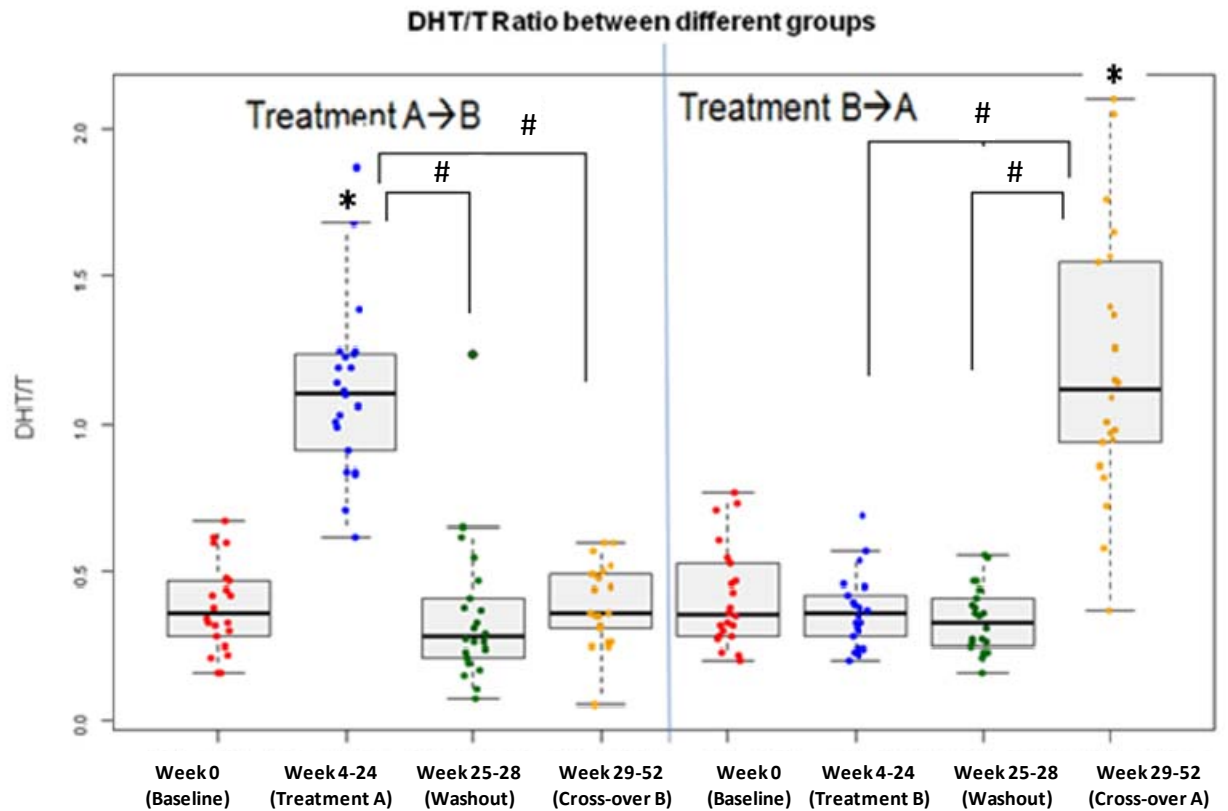
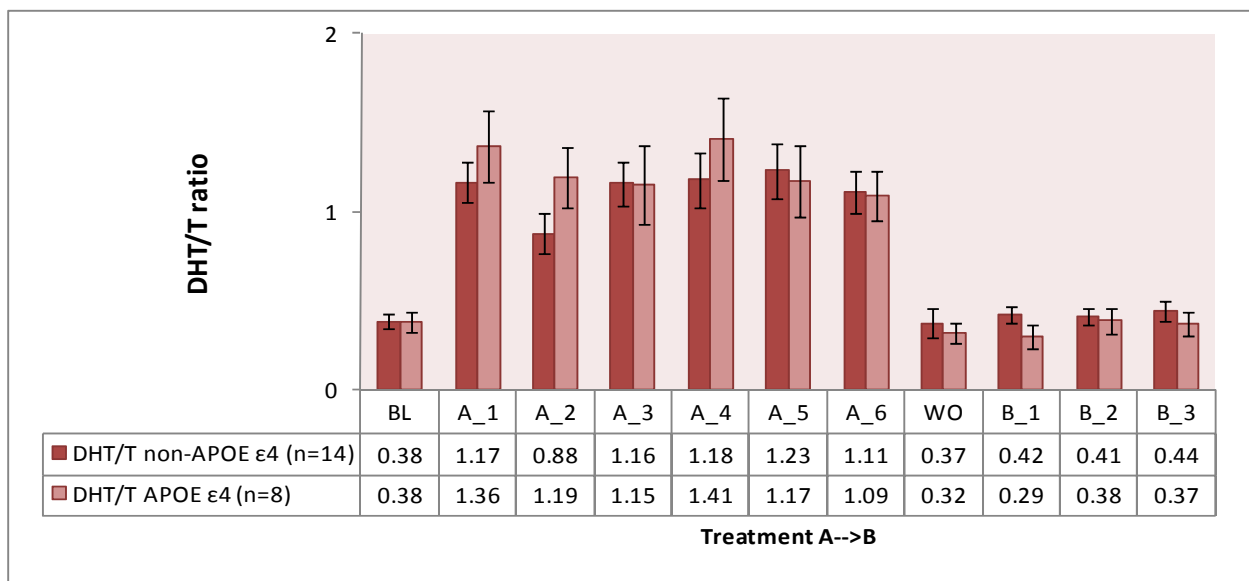


Figure 6.6 : Boxplots of serum DHT/Testosterone ratios for the two treatment arms at 4 different time points : Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are presented as median \pm quartiles. Values shown in the first treatment period are the averages from 6 visits and values in the second treatment period (Cross-over) are averages from 3 visits. Similar to results observed for testosterone and DHT levels, DHT to testosterone ratios were significantly increased in treatment A and cross-over A compared to other treatment periods in the two arms. * $p < 0.05$ in comparison to baseline of each arm of the treatments, # $p < 0.05$ in comparisons between other periods (shown by brackets).

(A)



(B)

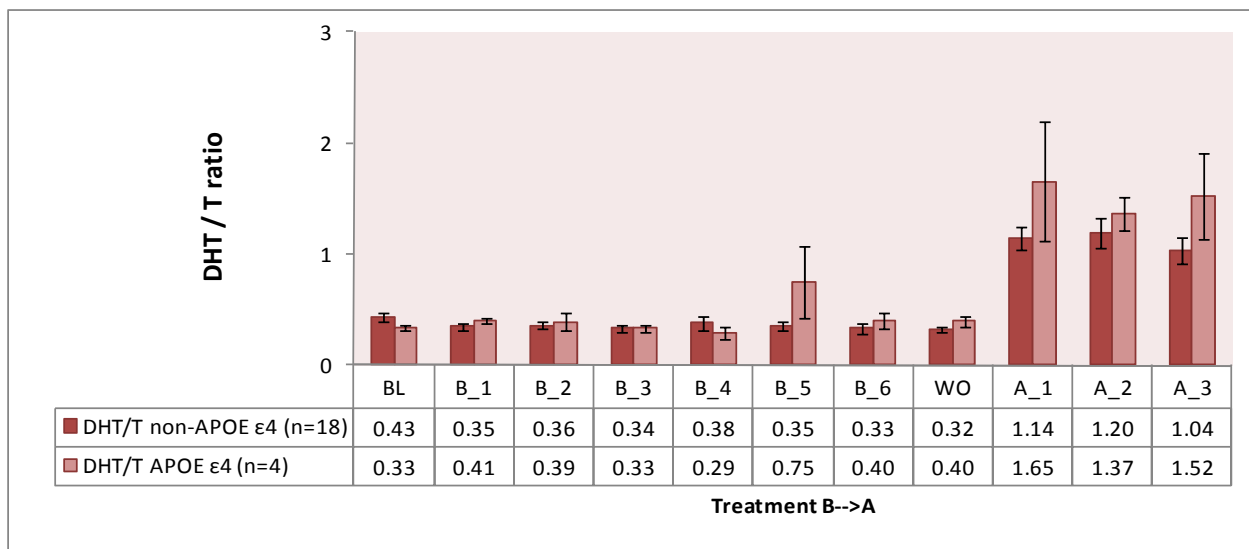


Figure 6.7 : Comparison of serum DHT/Testosterone ratios in APOE ε4 and non-APOE ε4 carriers (n=22) at 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values underneath the bars are the mean of each group. No significant differences were observed between the APOE ε4- and non-APOE ε4 groups in either treatment arm (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

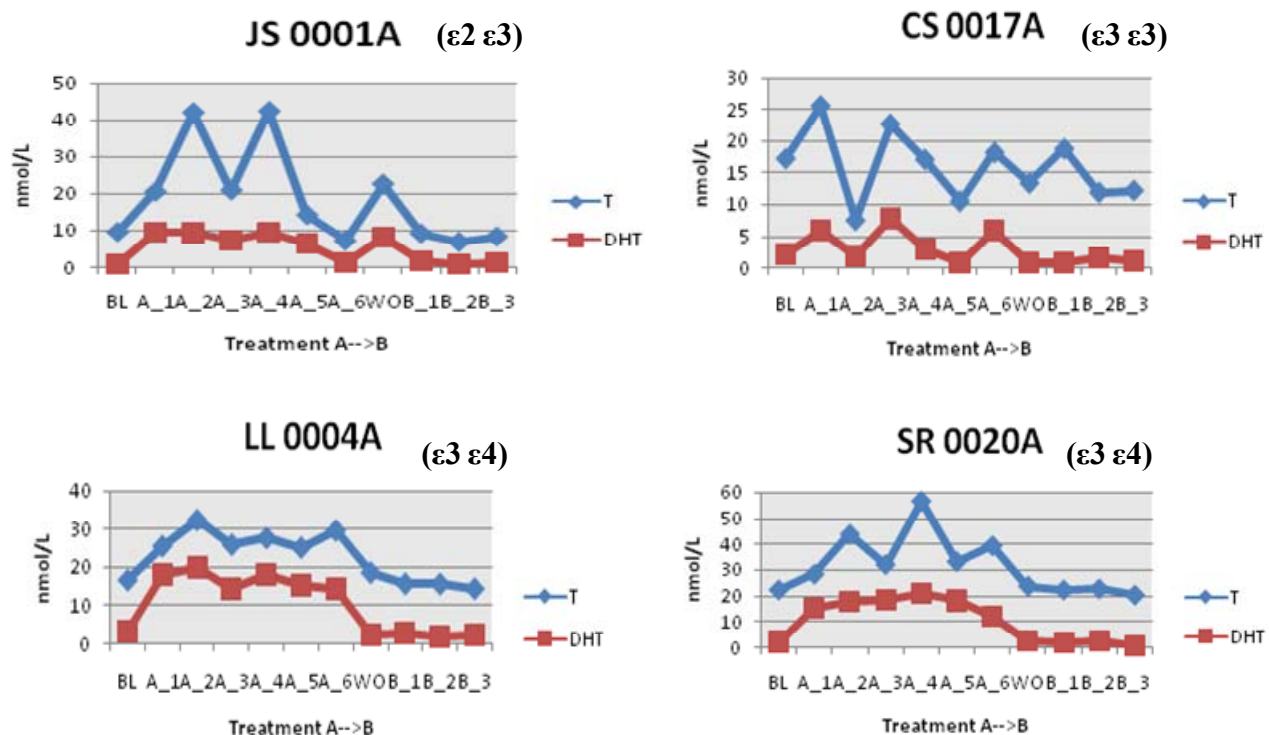


Figure 6.8 : Representative data of individual total testosterone and DHT levels in treatment A→B

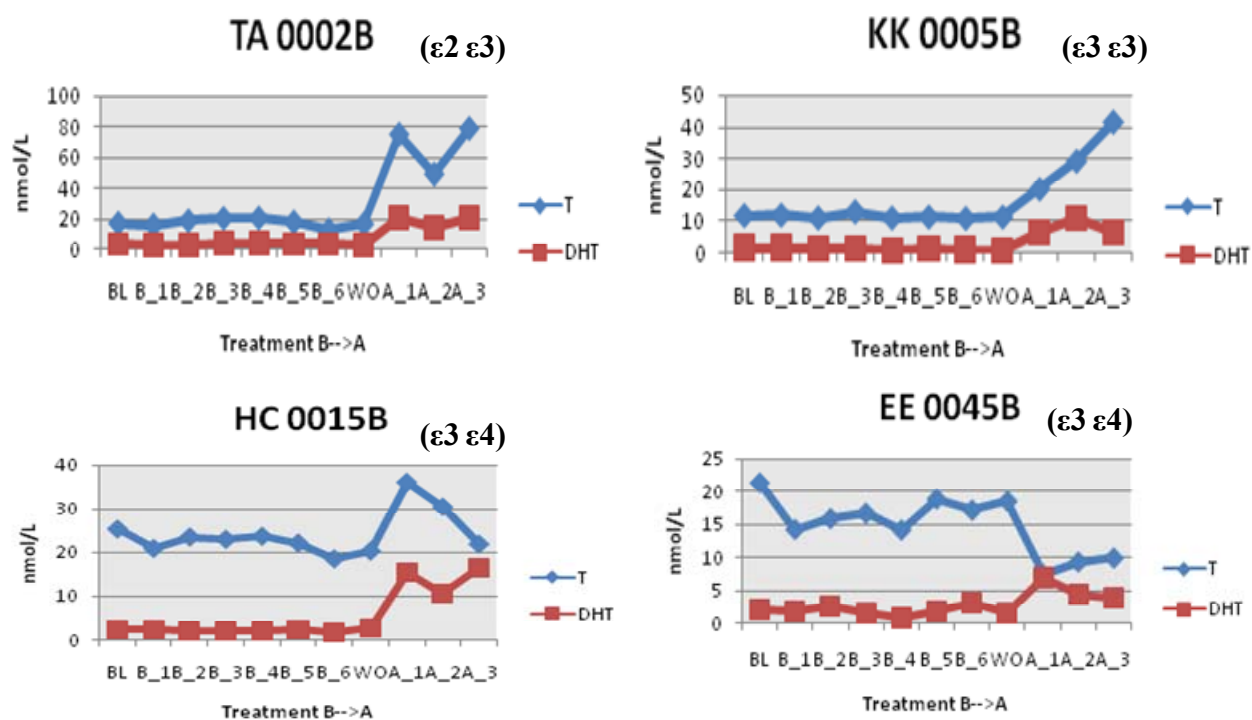


Figure 6.9 : Representative data of individual total testosterone and DHT levels in treatment B→A

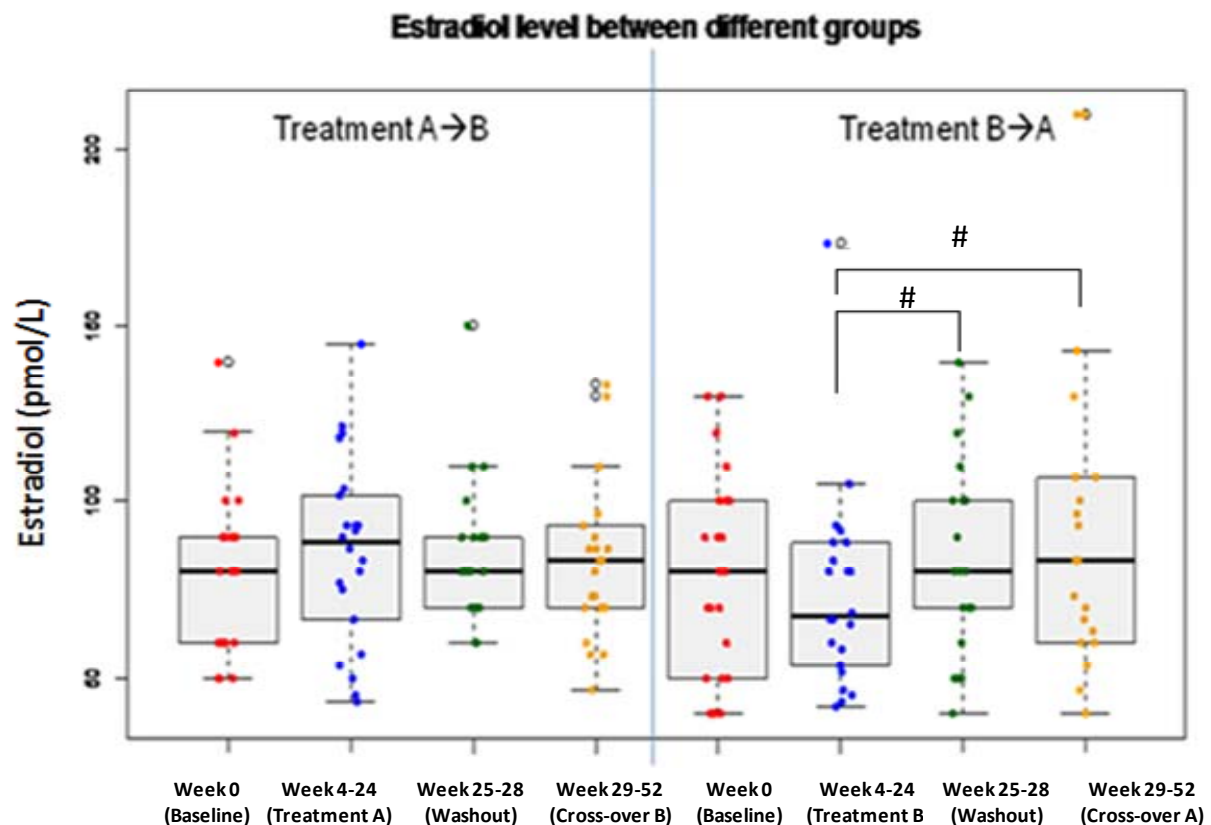
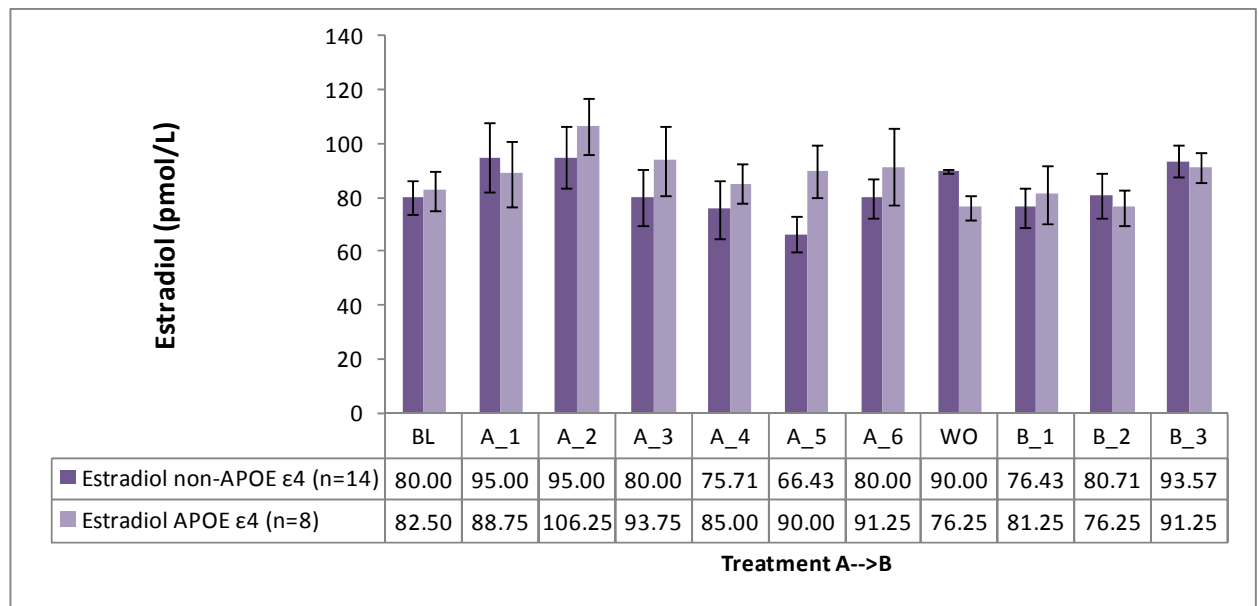


Figure 6.10 : Boxplots of serum Estradiol levels for both treatment arms at 4 different time points : Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are shown in pmol/L and are presented as median \pm quartiles. Treatment A values are the averages of measurements at 6 visits and values in the second treatment period (Cross-over) are averages from 3 visits. Significantly lower estradiol levels were measured during the placebo treatment (Treatment B) compared to washout and testosterone treatment (Cross-over A) in treatment arm B→A. # $p < 0.05$ in comparison to other treatment periods.

Changes in estradiol level were not found to be significant when comparing the different treatment stages of treatment arm A→B. However there were subtle changes seen in treatment B→A.

(A)



(B)

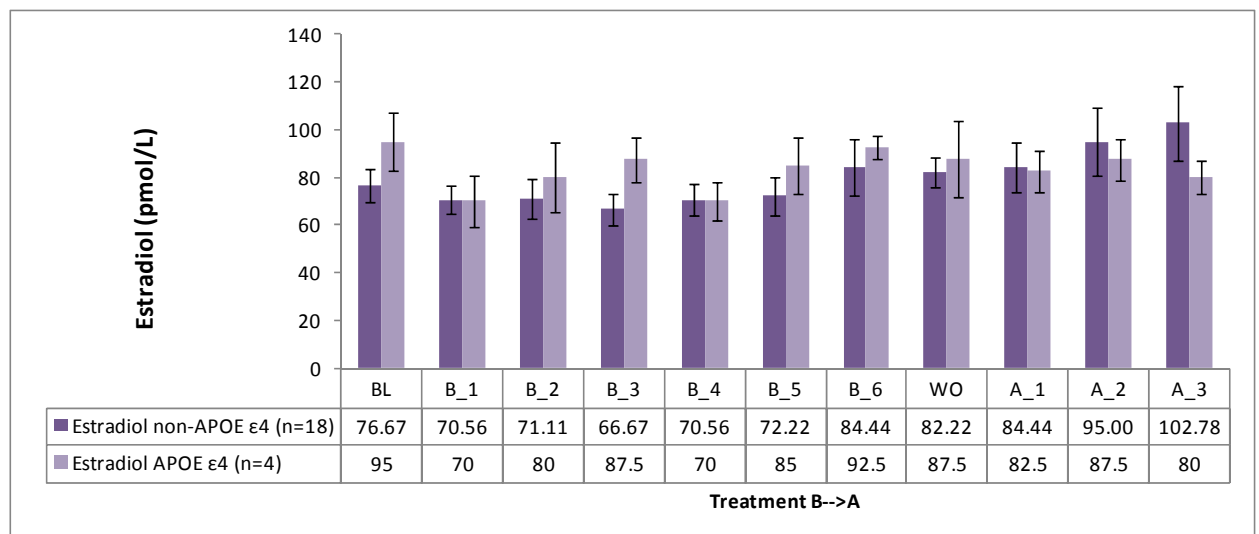


Figure 6.11 : Comparison of serum estradiol levels in APOE ε4 and non-APOE ε4 carriers (n=22) at the individual 11 visits of treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in pmol/L underneath the bars are the mean of each group. No differences were observed between the non-APOE ε4 and APOE ε4 groups of the two treatment arms. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

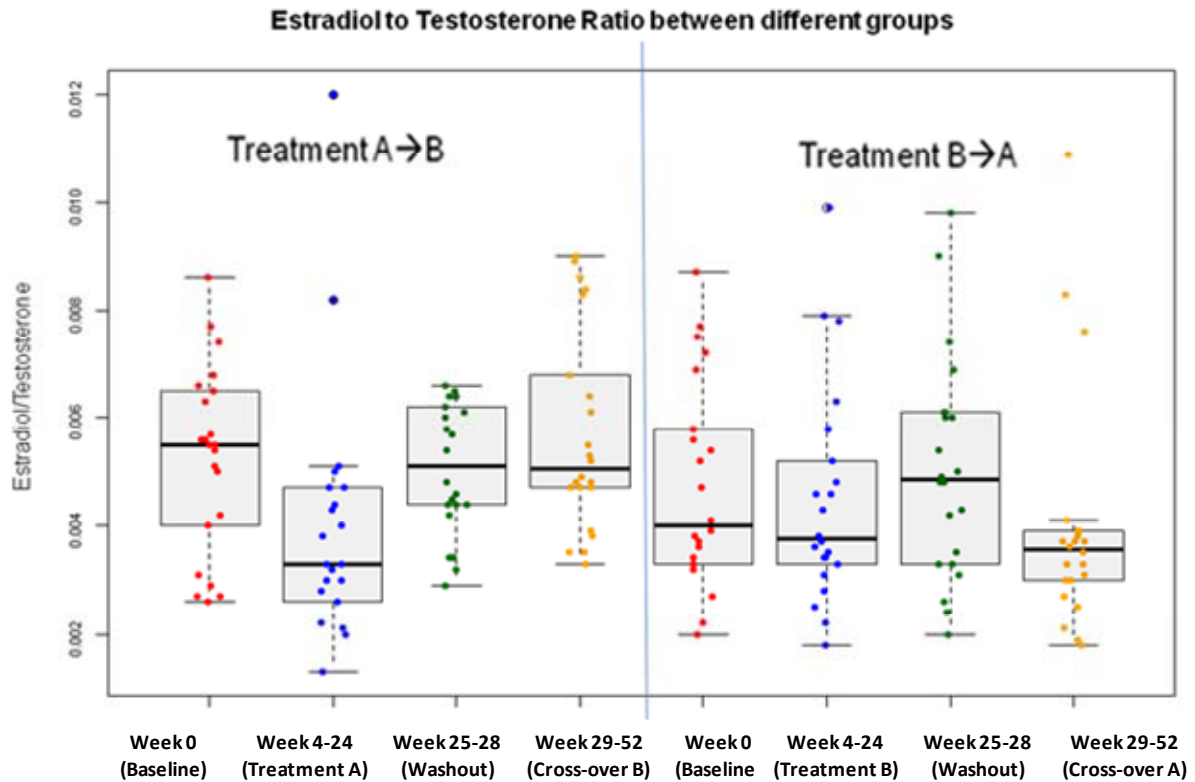
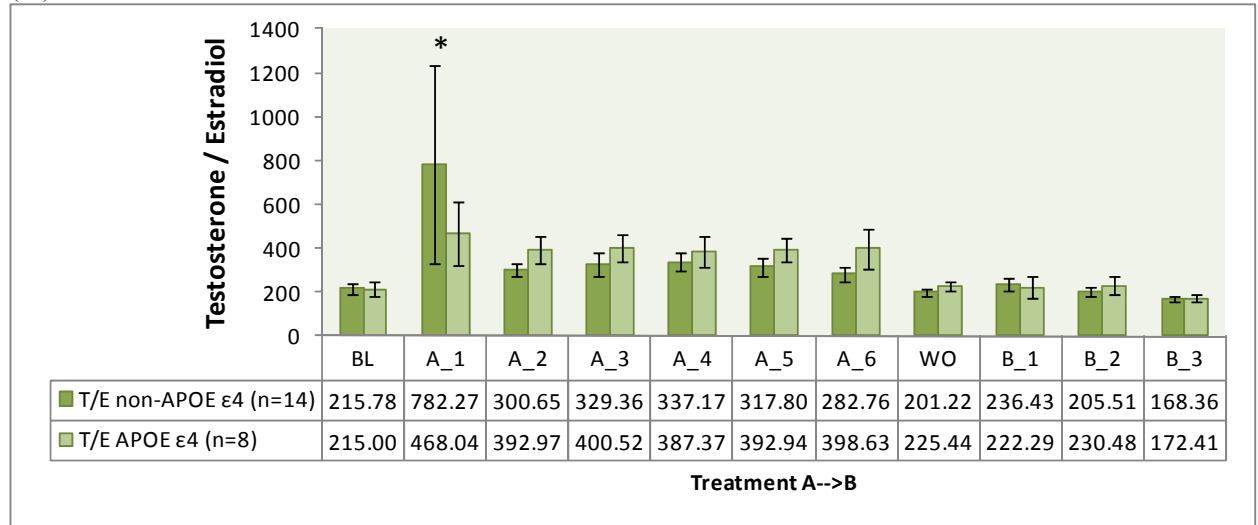


Figure 6.12 : Boxplot of serum Estradiol/Testosterone ratios for both treatment arms at the 4 different treatment stages: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are presented as median \pm quartiles. No significant differences were observed between any of the treatment stages of either of the arms. However, there was a trend towards decreased estradiol/testosterone ratios following testosterone treatment.

(A)



(B)

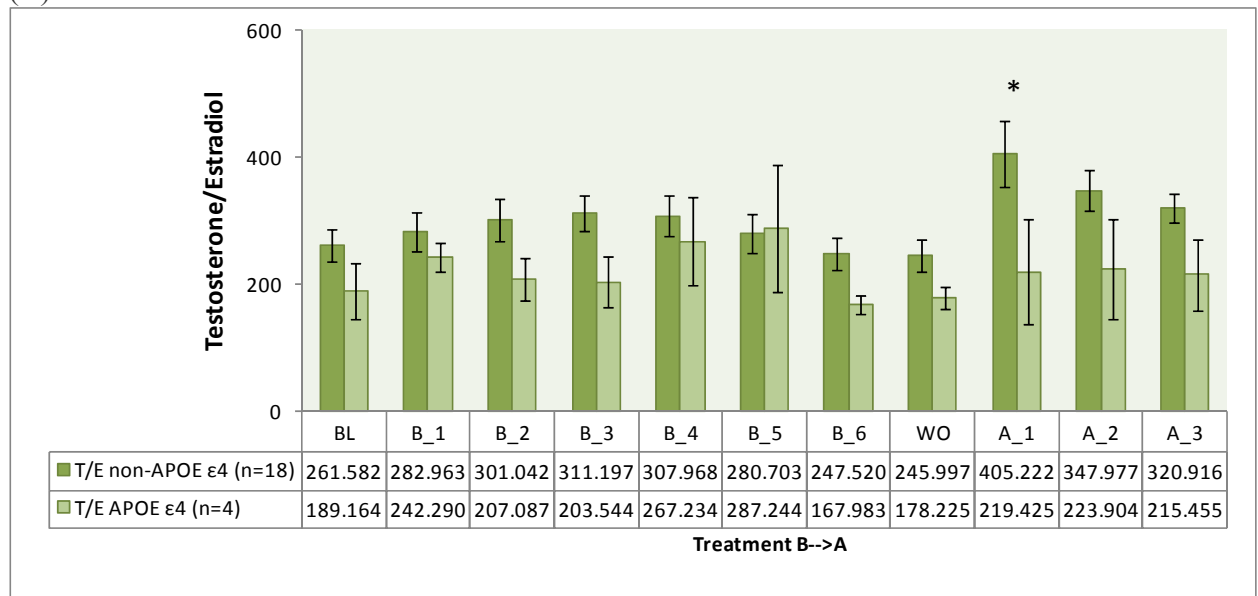


Figure 6.13 : Comparison of the testosterone/estradiol ratios in non-APOE ε4 and APOE ε4 carriers (n=22) at the 11 individual visits of treatment A→B (A) and treatment B→A (B). Values are presented as mean \pm standard deviation. Significant differences were observed between the non-APOE ε4 and APOE ε4 groups in both arm treatment within 4 weeks of testosterone treatment(*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. However it needs to be noted that there were only 4 APOE ε4 patients in the B→A treatment group

6.4.2 Effects of testosterone administration on Luteinizing Hormone (LH) levels in the plasma

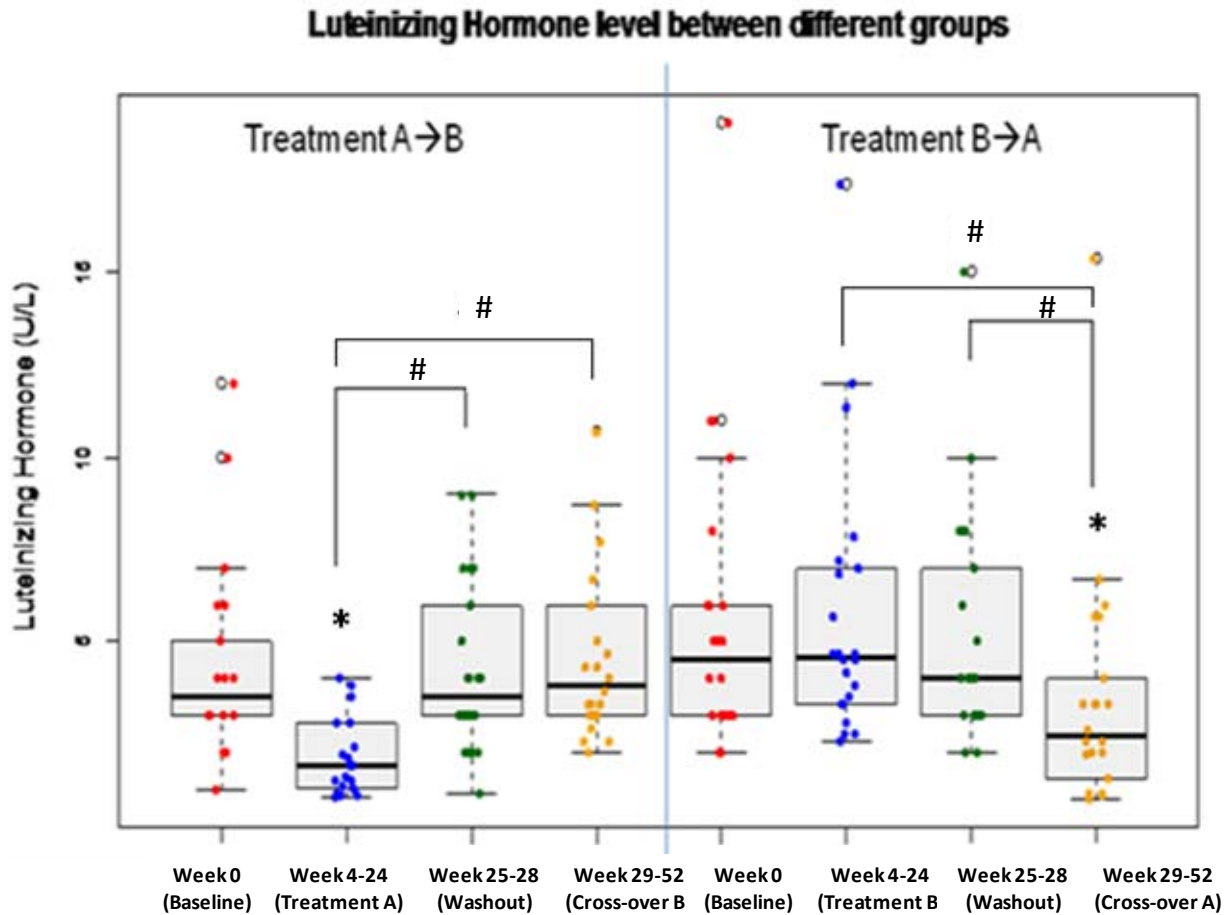
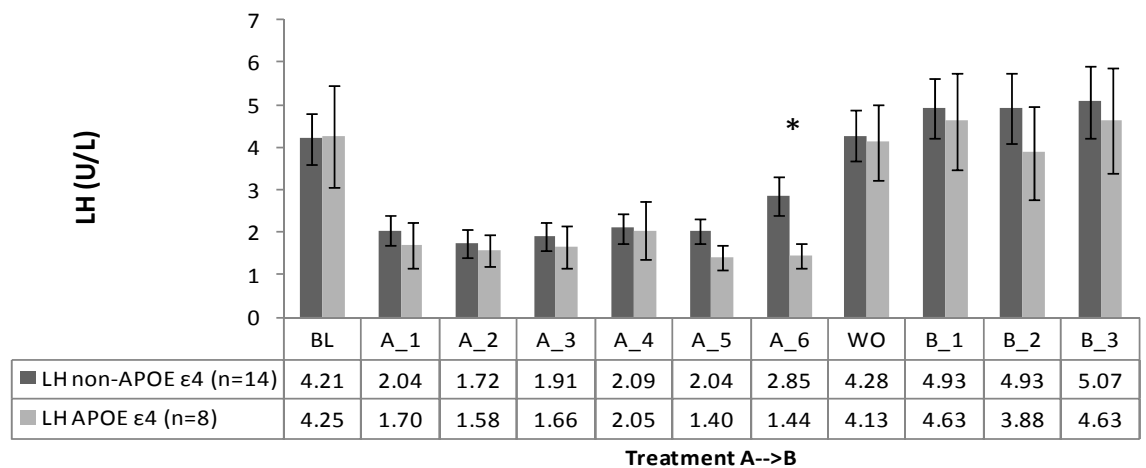


Figure 6.14 : Boxplots of serum Luteinizing Hormone (LH) levels for both treatment arms measured at the 4 different treatment stages: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in U/L and are presented as median \pm quartiles. Values in the Treatment stages are average serum levels from 6 visits and values in the second (Cross-over) treatment period are the averages from 3 visits. LH levels were significantly decreased in treatment A and cross-over A (ie testosterone treatment times) compared to all other treatment periods in both arms. * $p < 0.05$ in comparison to baseline, # $p < 0.05$ in comparisons between other treatment periods.

(A)



(B)

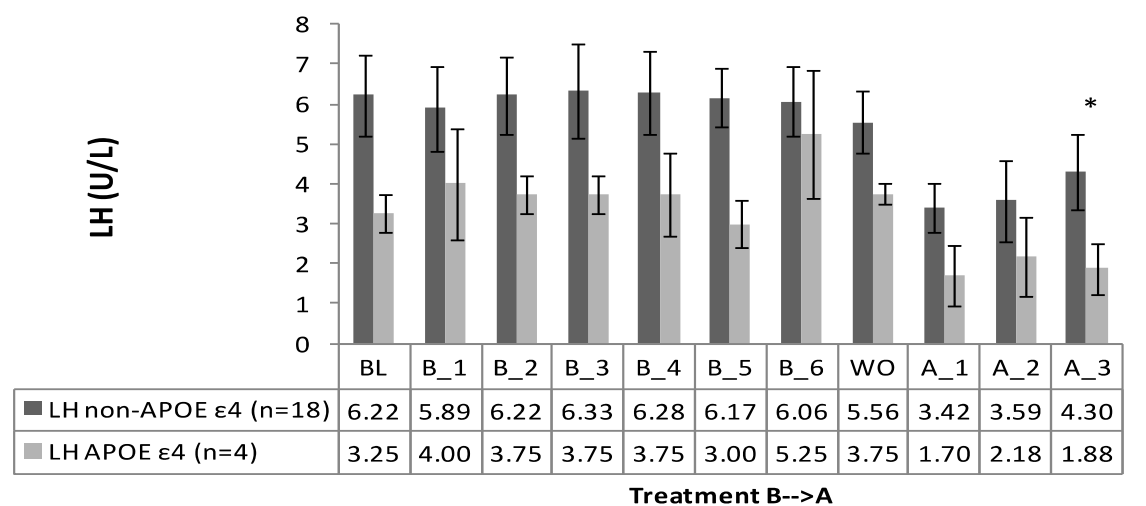


Figure 6.15 : Comparison of serum LH levels in non-APOE ε4 and APOE ε4carriers (n=22) at the 11 individual visits of treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in U/L underneath the bars are the mean of each group. Significant differences were observed between the non-APOE ε4 and APOE ε4 groups of both treatment arms at the end of testosterone treatment (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

6.4.2 Effects of testosterone administration on plasma lipid profiles of the participants.

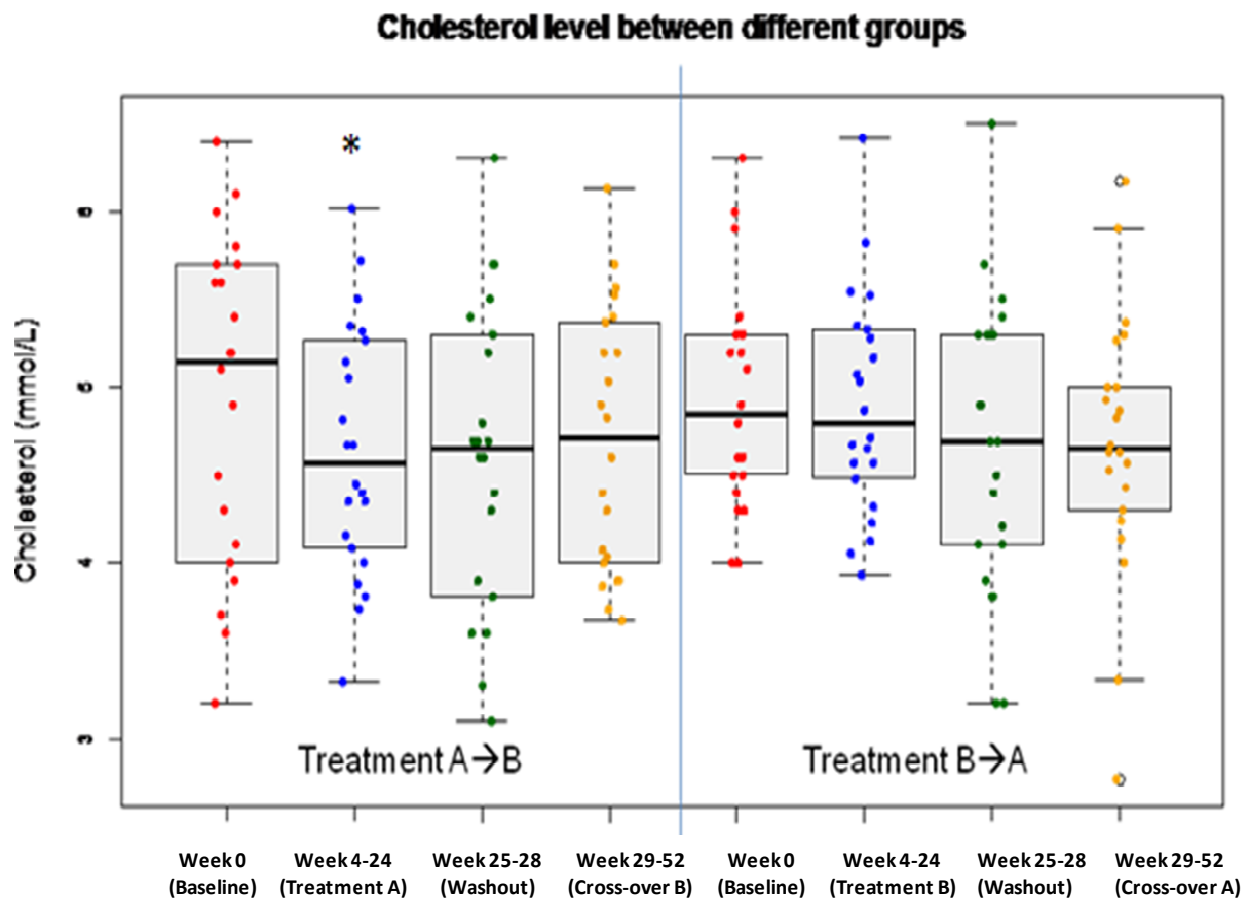
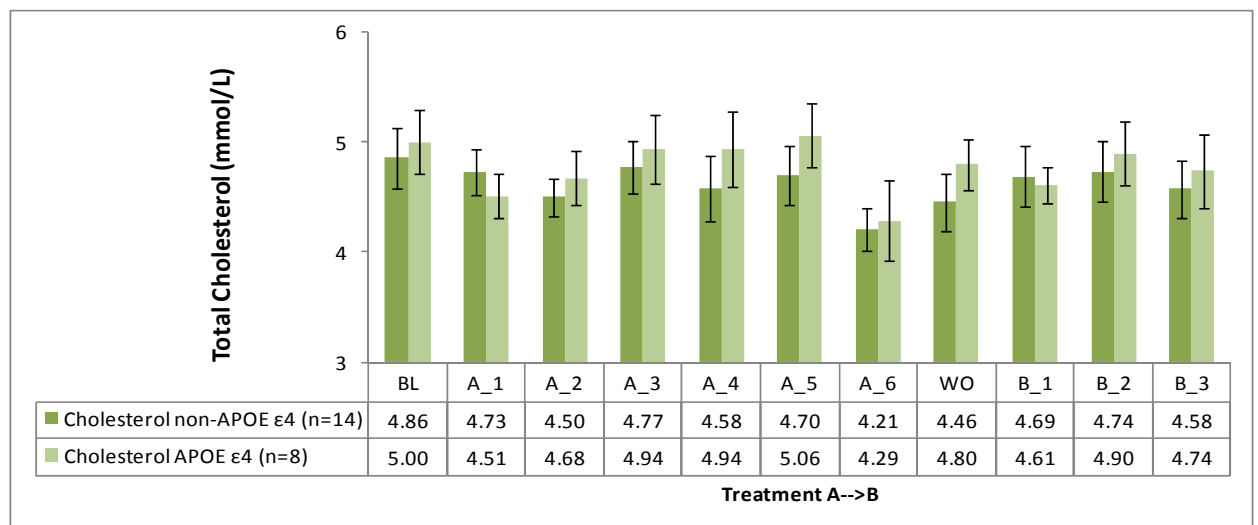


Figure 6.16 : Boxplots of Total serum Cholesterol levels for both treatment arms at the 4 different treatment stages: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in mmol/L and are presented as median \pm quartiles. Values in the Treatment stages are average levels from 6 visits and values in the second (Cross-over) treatment period are the averages from 3 visits. There was a significant decrease in total cholesterol levels during testosterone treatment in treatment arm A→B. * $p < 0.05$ in comparison to baseline in treatment arm A→B.

(A)



(B)

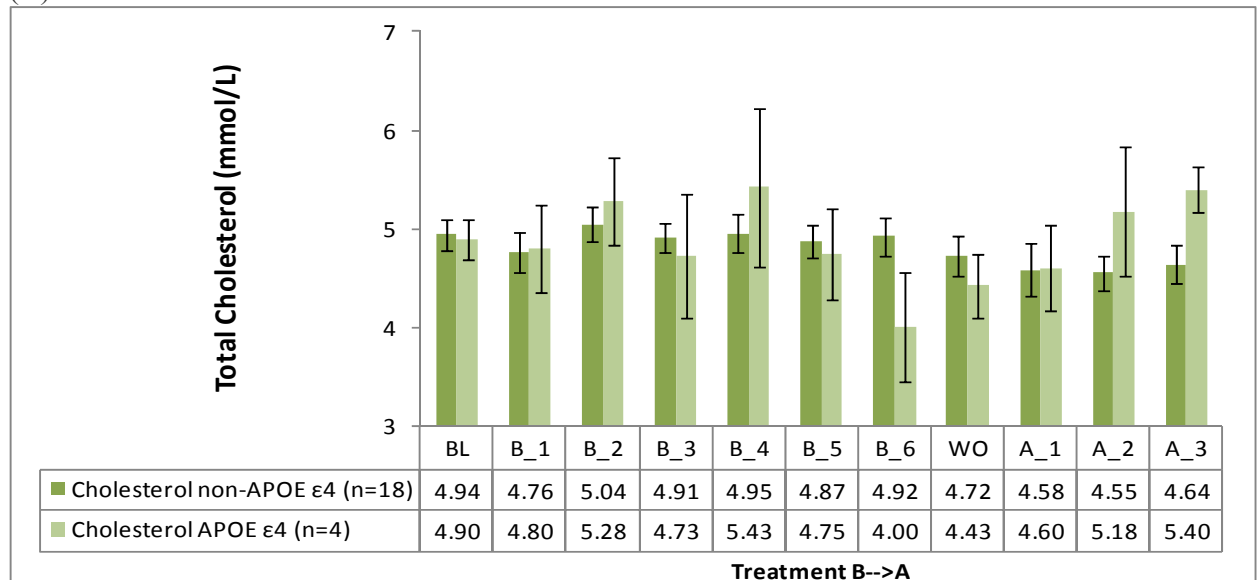


Figure 6.17: Comparison of total cholesterol levels in non-APOE ε4 and APOE ε4 carriers: average results from each of the 11 visits for treatment arms A→B (A) and B→A (B). The bars represent the mean \pm standard deviation. Values in mmol/L underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ε4 and APOE ε4 groups of the two treatment arms. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period, (n=22).

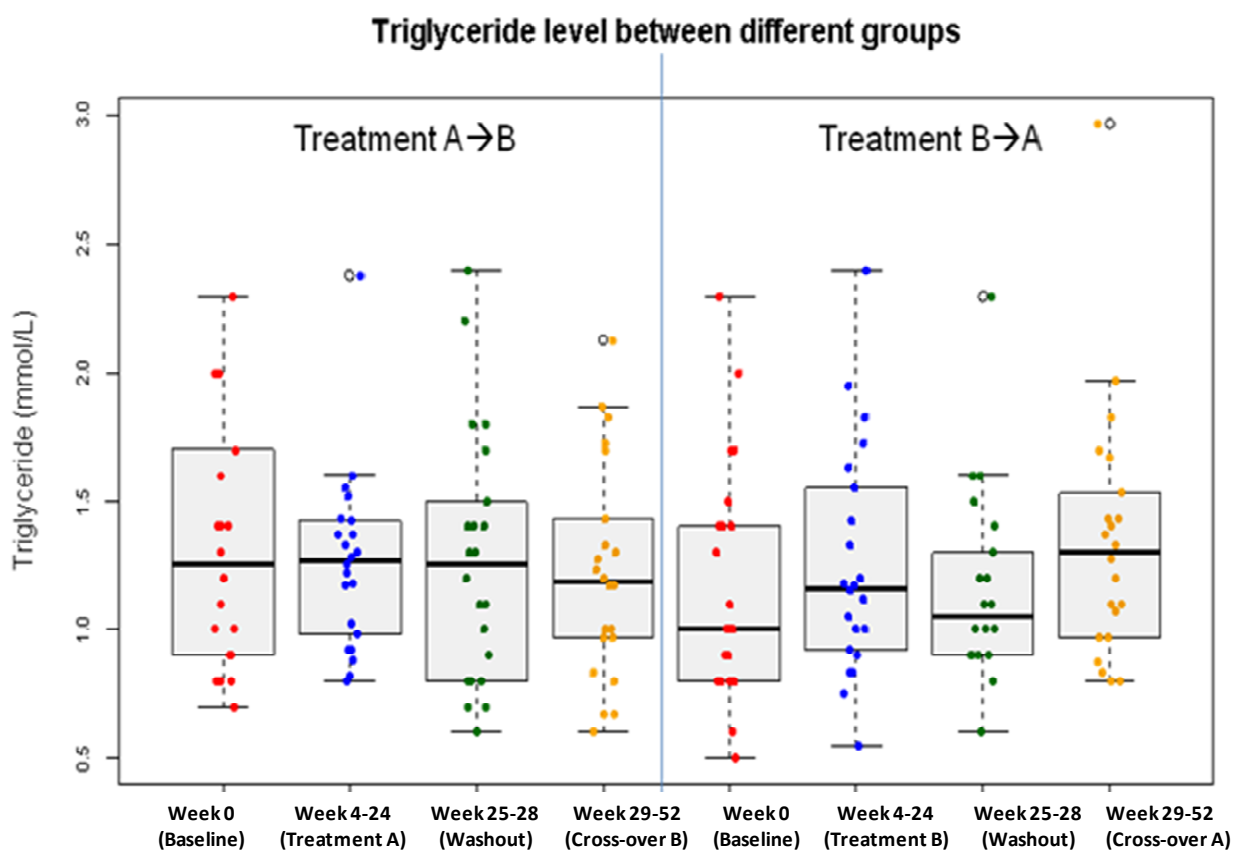
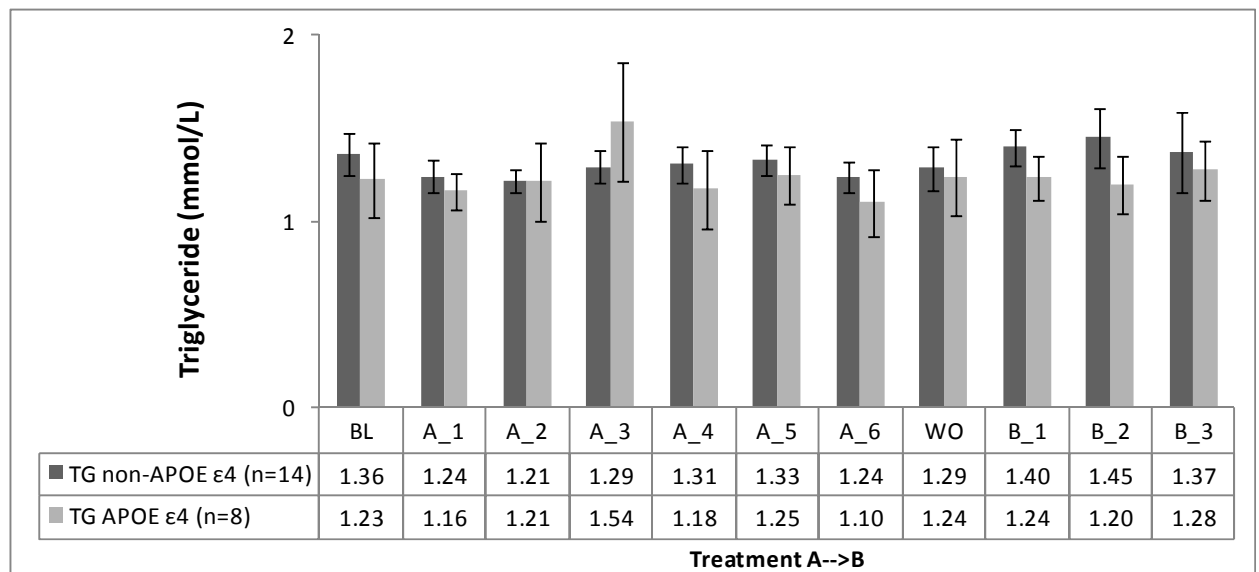


Figure 6.18: Boxplots of serum Triglyceride (TG) levels for the two treatment arms at the 4 different treatment stages: Week 0 (Baseline), Week 4-24 (First Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in mmol/L and are presented as median \pm quartiles. The values in the first treatment stages are the average levels from 6 measurements over the treatment period and values in the second treatment (Cross-over) period are the average of 3 measurements. There were no significant differences observed in either of the treatment groups.

(A)



(B)

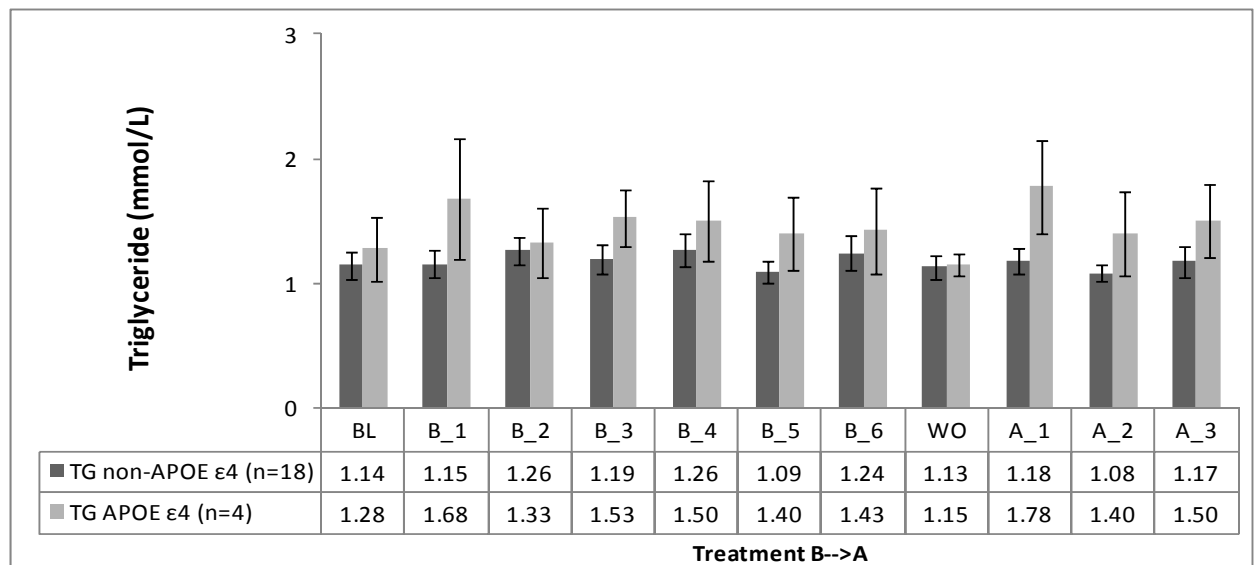


Figure 6.19 : Comparison of serum triglyceride (TG) levels in non-APOE ε4 and APOE ε4 carriers: average results from the 11 individual visits in treatment arms A→B (A) and treatment B→A (B). The bars represent the mean ± standard deviation. Values in mmol/L underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ε4 and APOE ε4 groups in either arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment

period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

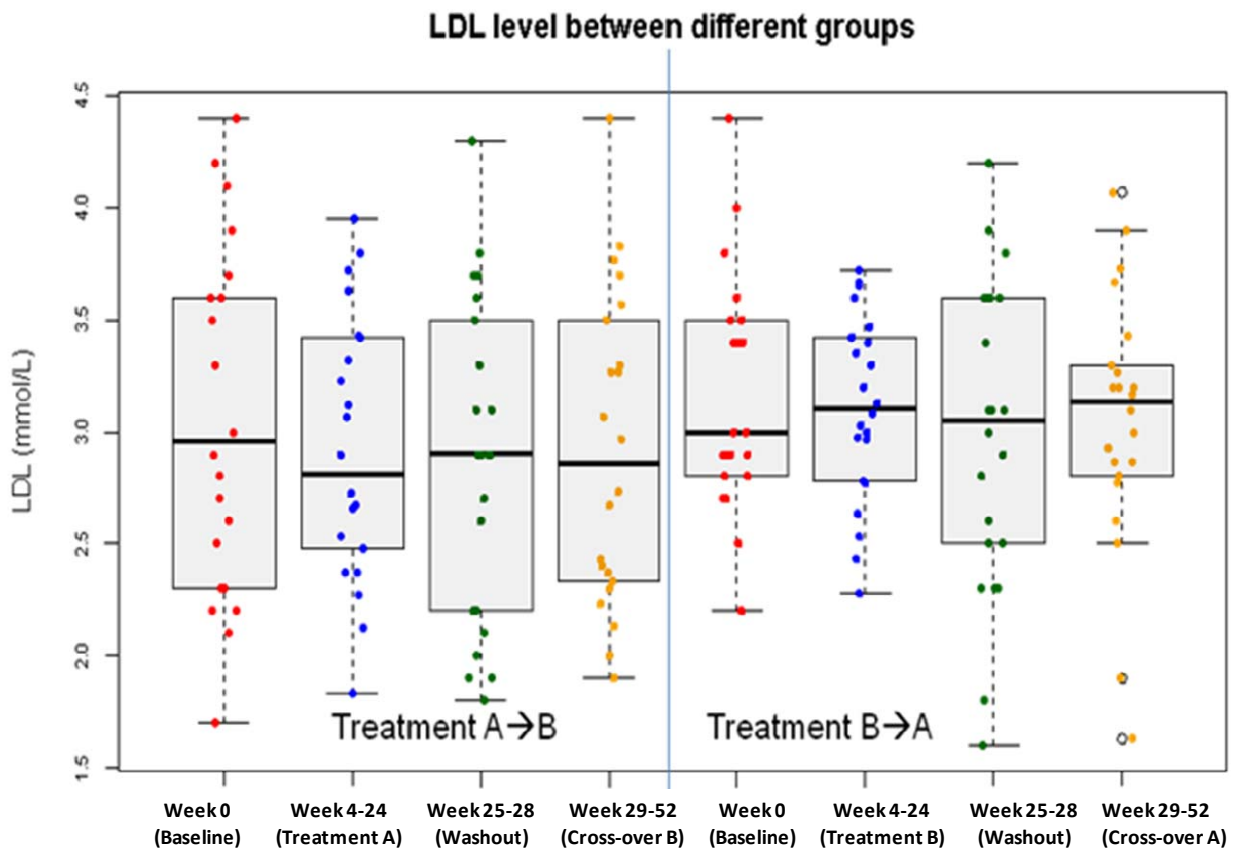
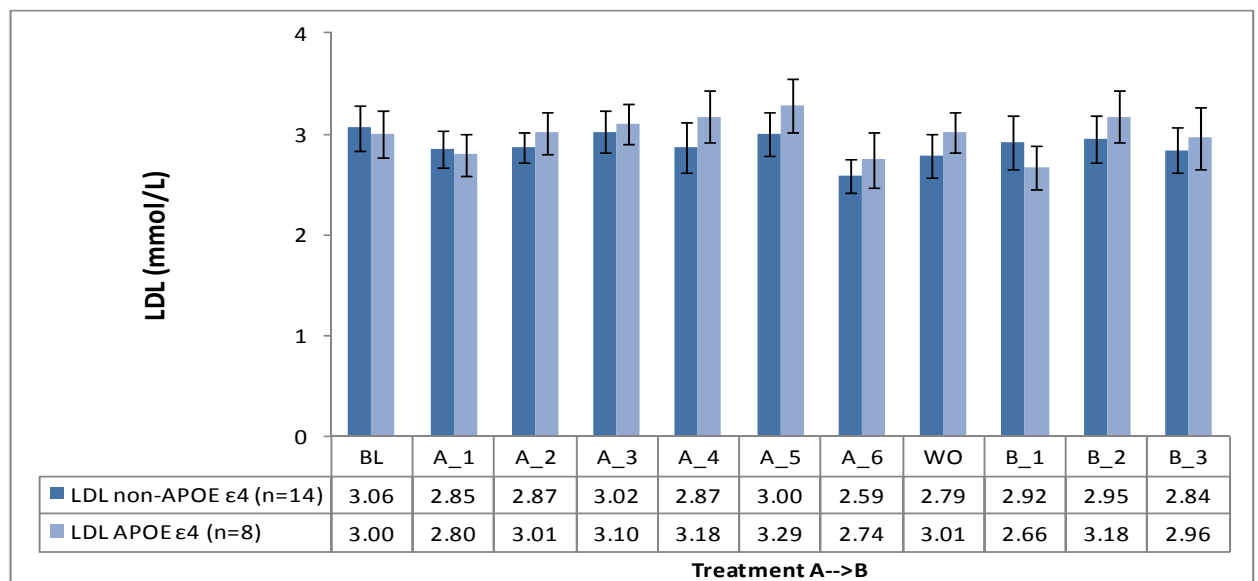


Figure 6.20 : Boxplots of serum Low Density Lipoprotein (LDL) levels for the two treatment arms showing averages from the 4 different treatment periods: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in mmol/L and are presented as median \pm quartiles. Values shown in the first treatment period are the average results from blood samples from 6 visits, and values in the second (Cross-over) treatment period were averaged from 3 visits. There were no differences observed between any of the 4 stages of either arm.

(A)



(B)

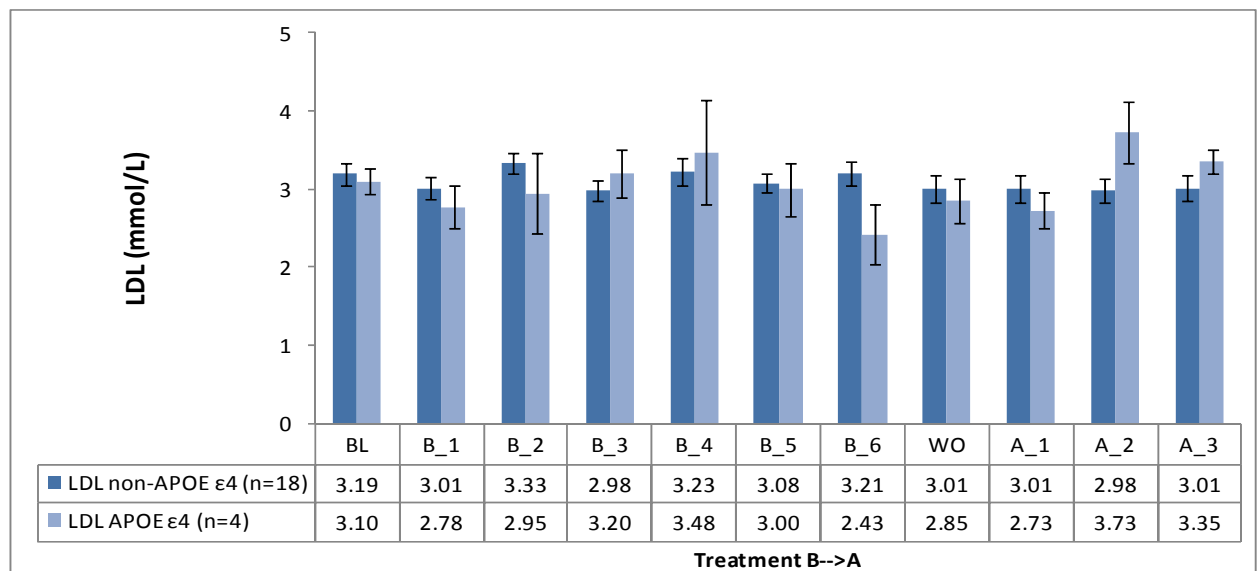


Figure 6.21 : Comparison of LDL levels in non-APOE ε4 and APOE ε4 carriers at each of the 11 visits of treatment arms A→B (A) and B→A (B). The bars represent the mean \pm standard deviation. Values in mmol/L underneath the bars are the mean of each group. No significant differences were observed between non-APOE ε4 and APOE ε4 groups of either arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

Overall, no significant changes in LDL level were observed between the stages of either of the treatment arms. However, in treatment arm A→B, we found a slight decrease in LDL levels in the non-APOE ε4 group following testosterone treatment. After testosterone treatment, LDL levels returned back to that seen at baseline level. Interestingly, in treatment B→A, we found a slight increase of LDL following testosterone treatment in the APOE ε4 group. (Figures 6.20 and 6.21).

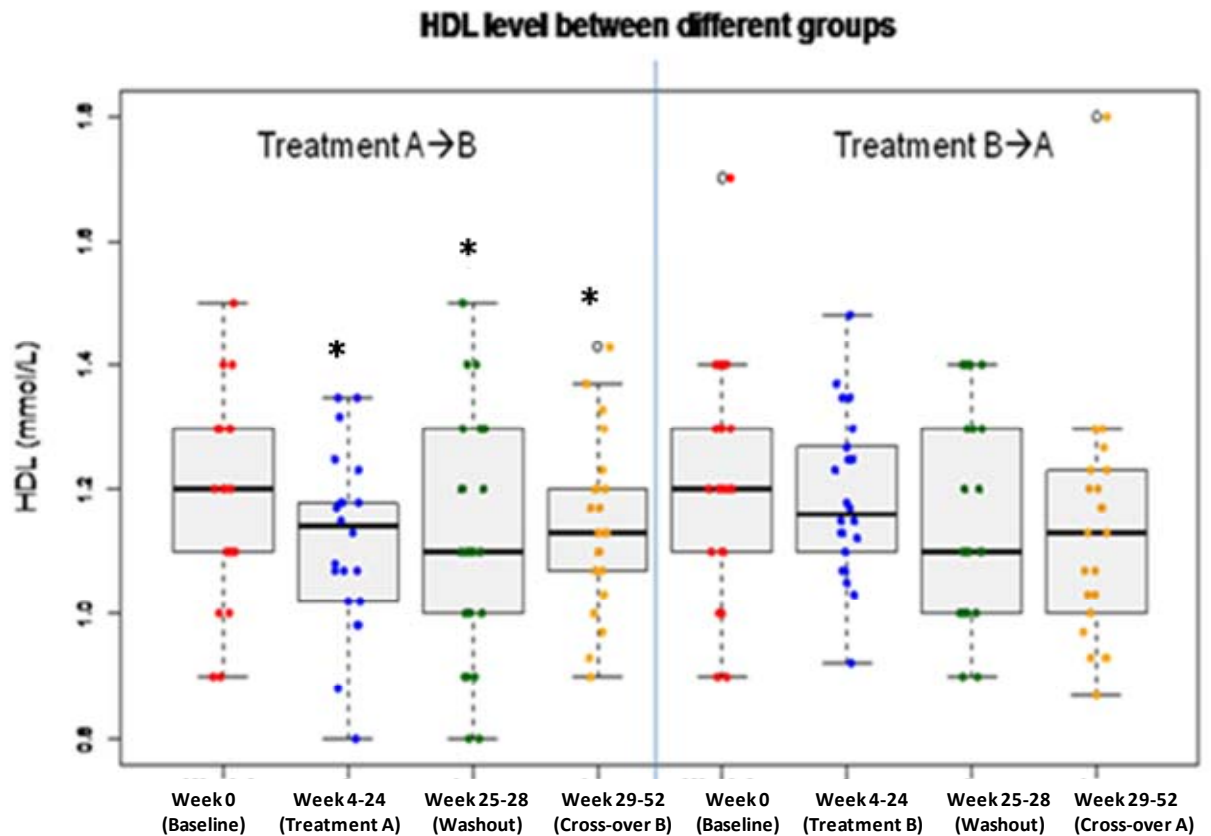
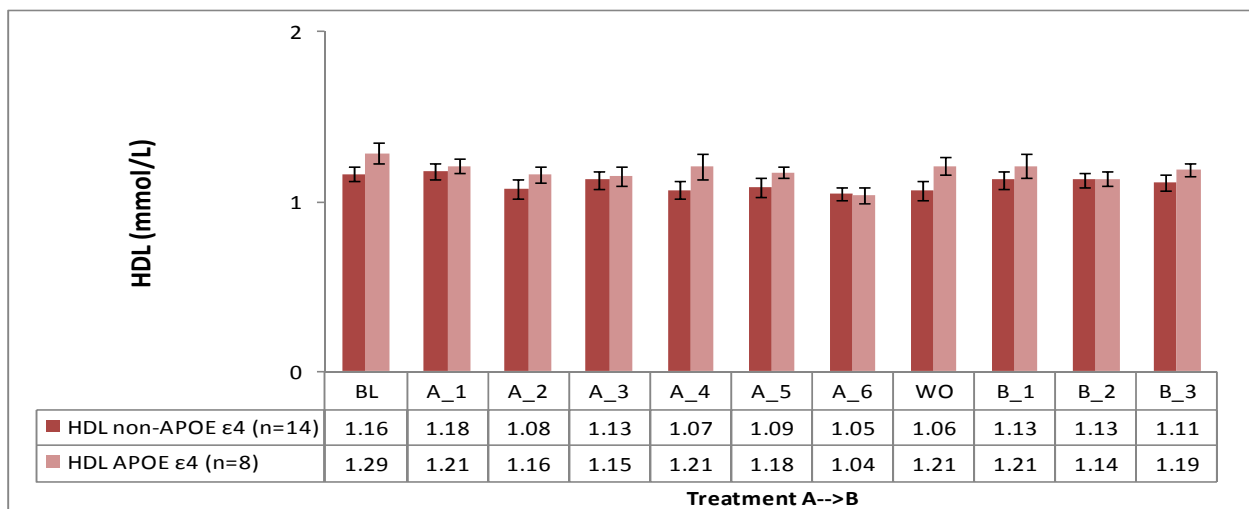


Figure 6.22 : Boxplots of High Density Lipoprotein (HDL) levels for both treatment arms during the 4 different treatment periods: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in mmol/L and presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the second (Cross-over) treatment are the averages from 3 visits. Compared to baseline levels, there were significant decreases in HDL levels at all other stages in treatment arm A→B. * $p < 0.05$ in comparison to baseline, however there were no significant differences in the B→A arm.

(A)



(B)

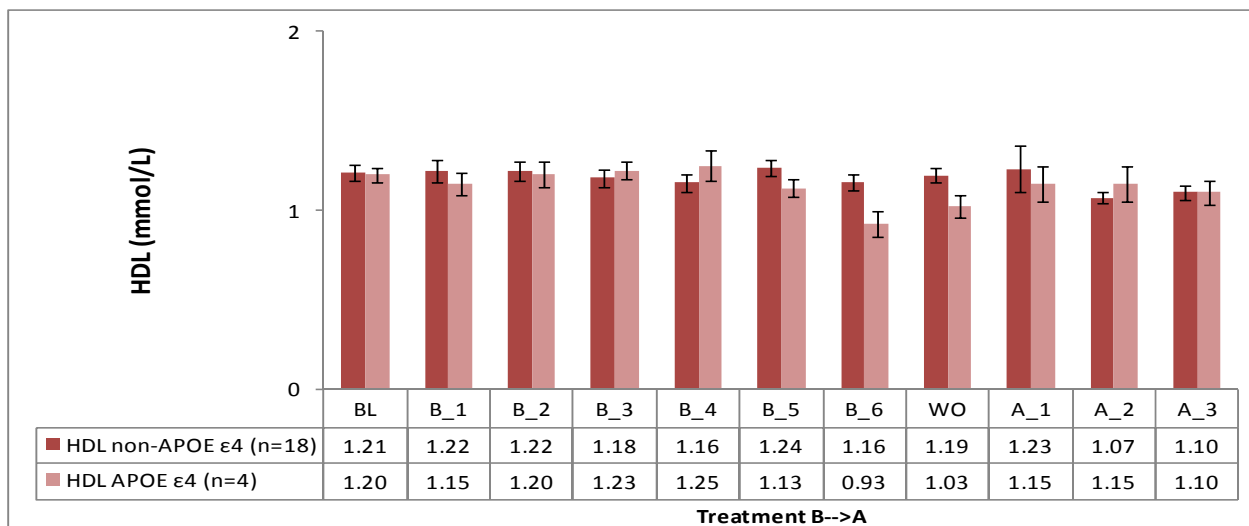


Figure 6.23 : Comparison of HDL levels in APOE ε4 and non-APOE ε4 carriers at the 11 individual visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in mmol/L underneath the bars are the mean of each group. There were no significant differences observed between the non-APOE ε4 and APOE ε4 groups of either arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

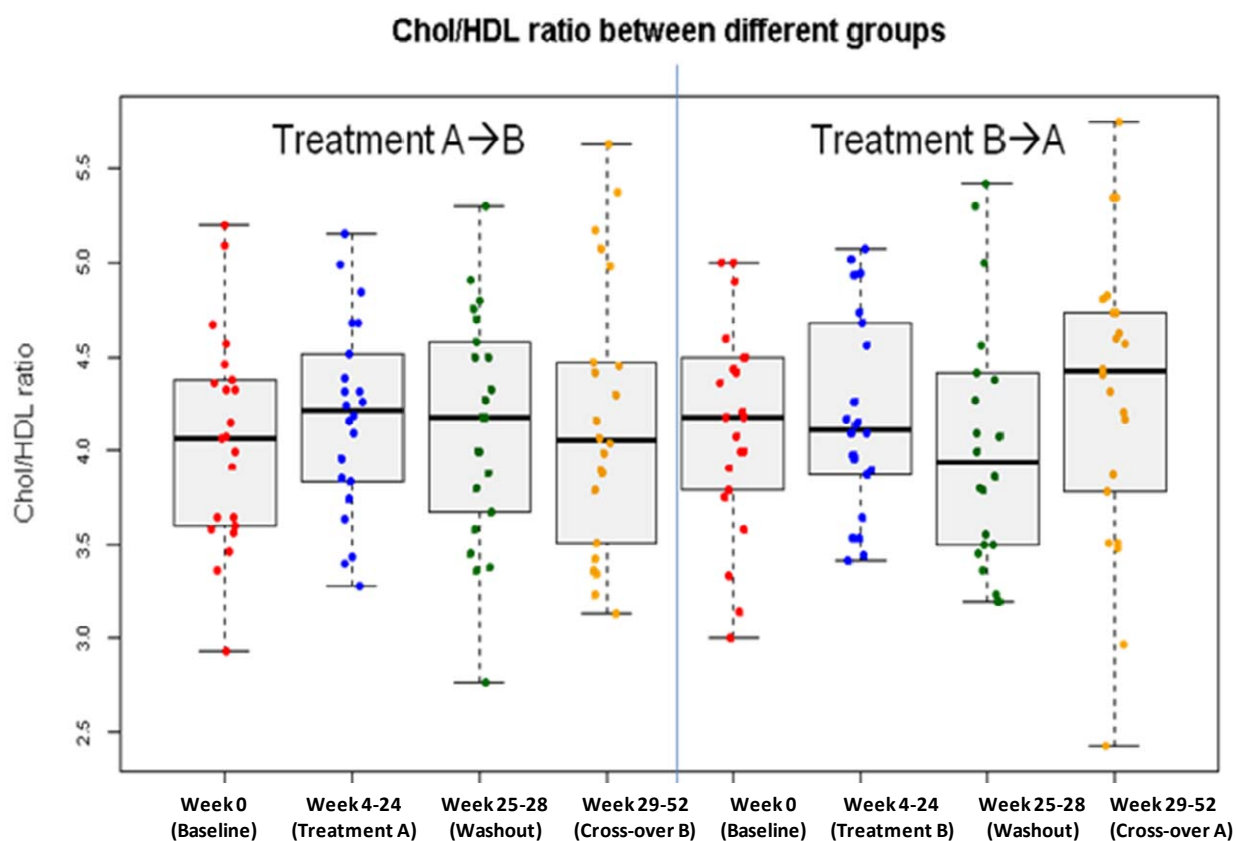
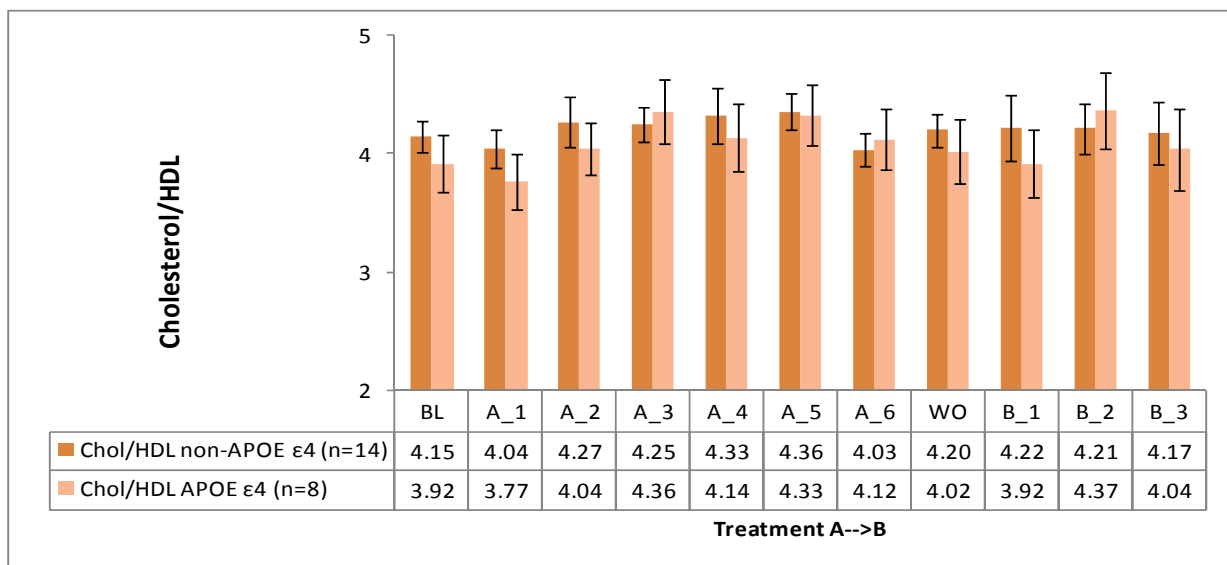


Figure 6.24 : Boxplots of Cholesterol/HDL ratios for each of the 4 stages of treatment A→B and treatment B→A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the second treatment (Cross-over) stages are the average results from 3 visits. There were no differences observed in either treatment arm.

(A)



(B)

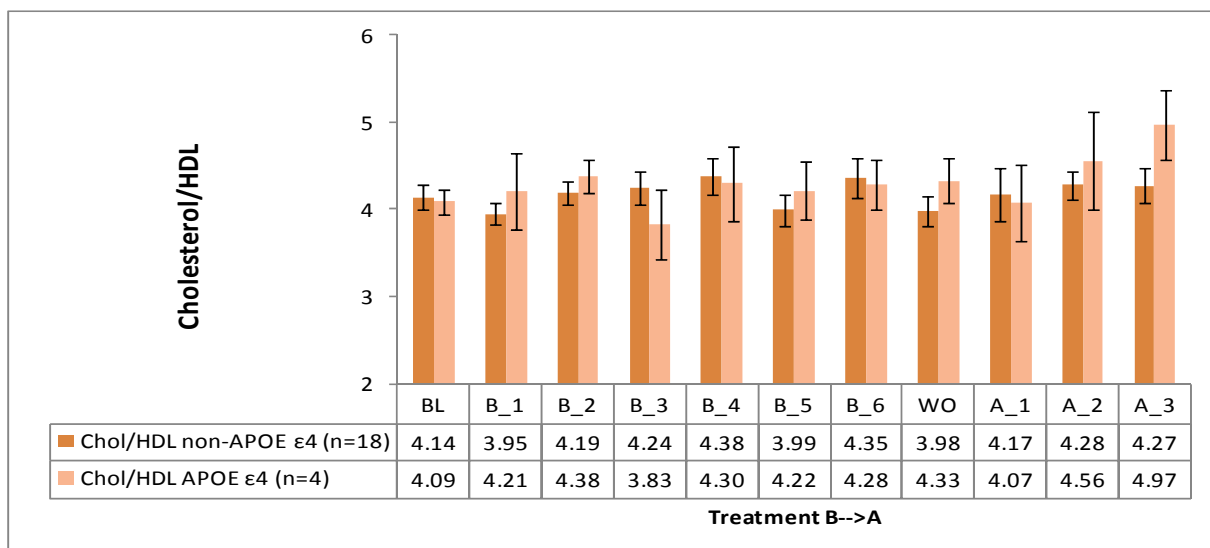


Figure 6.25 : Comparison of plasma cholesterol/HDL ratios in non-APOE ϵ 4 and APOE ϵ 4 carriers at each of the individual 11 visits of treatments A→B (A) and B→A (B). The bars represent the mean \pm standard deviation. Values underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ϵ 4 and APOE ϵ 4 groups of either treatment arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

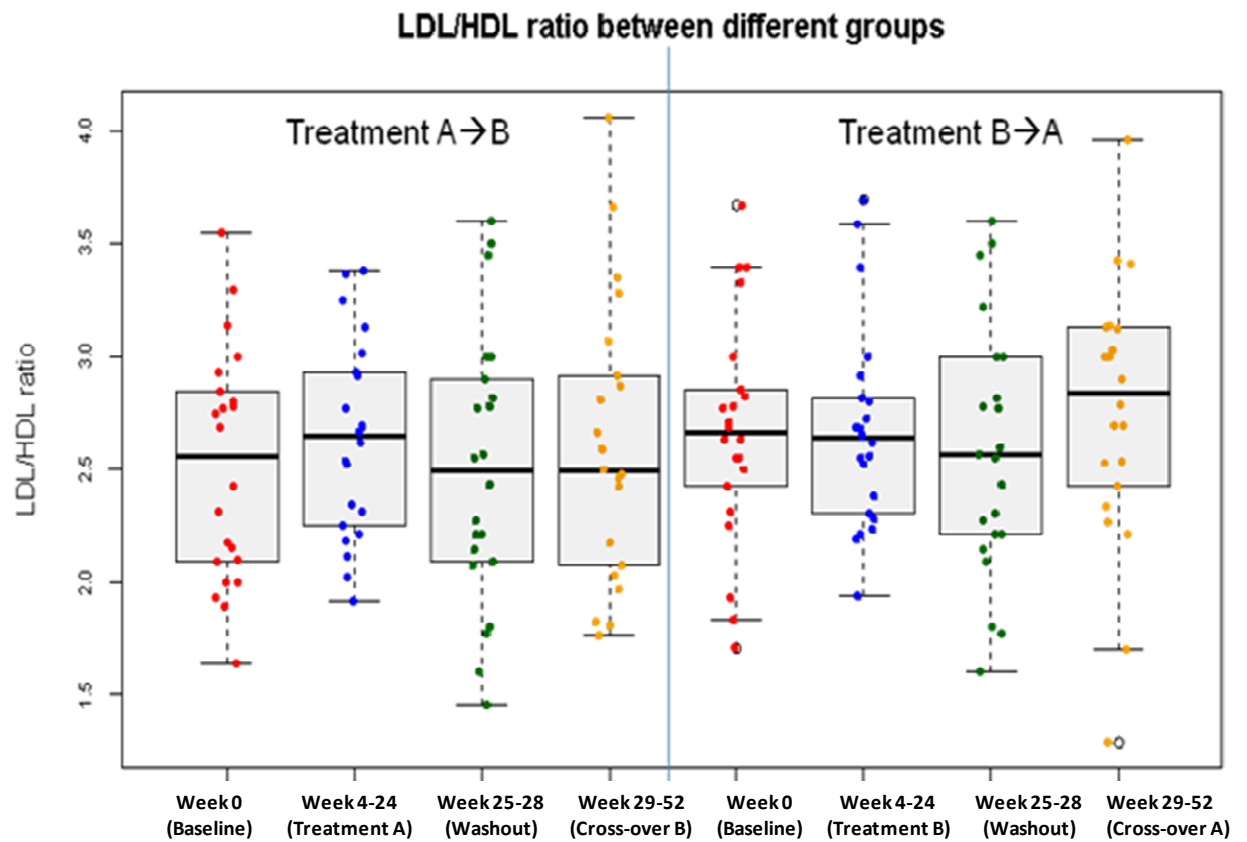
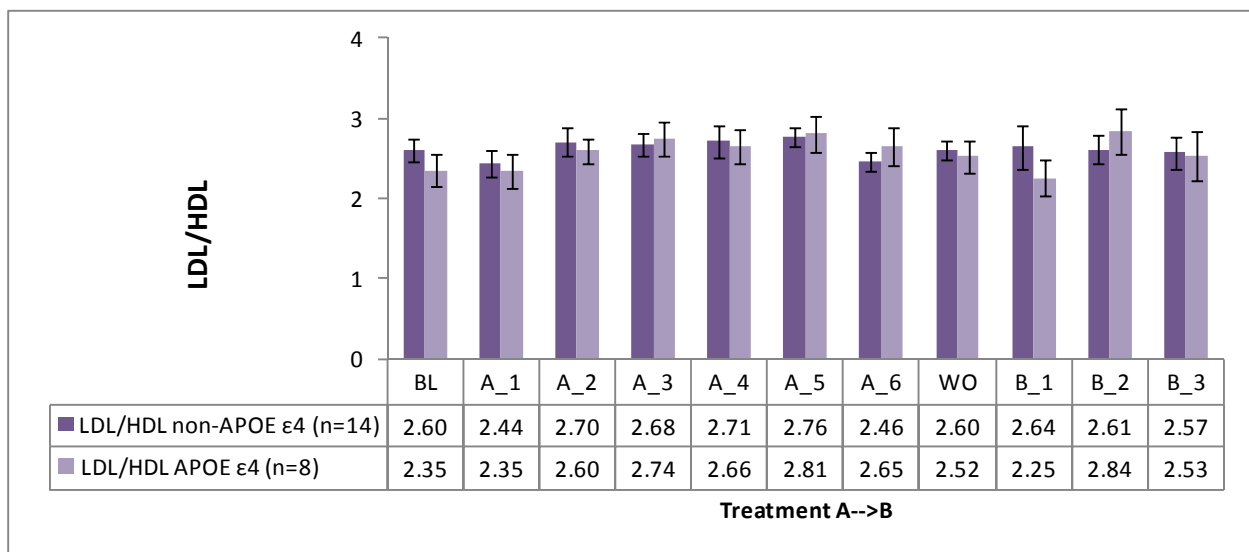


Figure 6.26: Boxplots of LDL/HDL ratios at each treatment stage in treatment A→B and treatment B→A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the Cross-over (second treatment) stages are the averages from 3 visits. There were no differences observed between the stages of either treatment group.

(A)



(B)

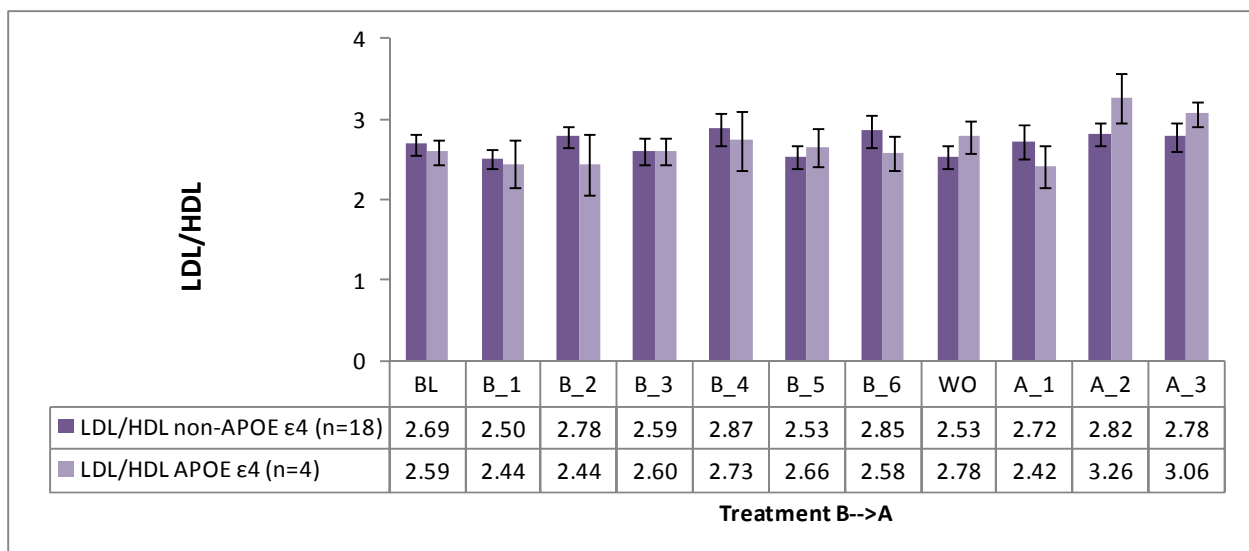


Figure 6.27: Comparison of LDL/HDL ratios in non-APOE ε4 and APOE ε4 at each of the individual 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ε4 and APOE ε4 groups of either treatment arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

6.4.3. Effects of testosterone on plasma A β levels

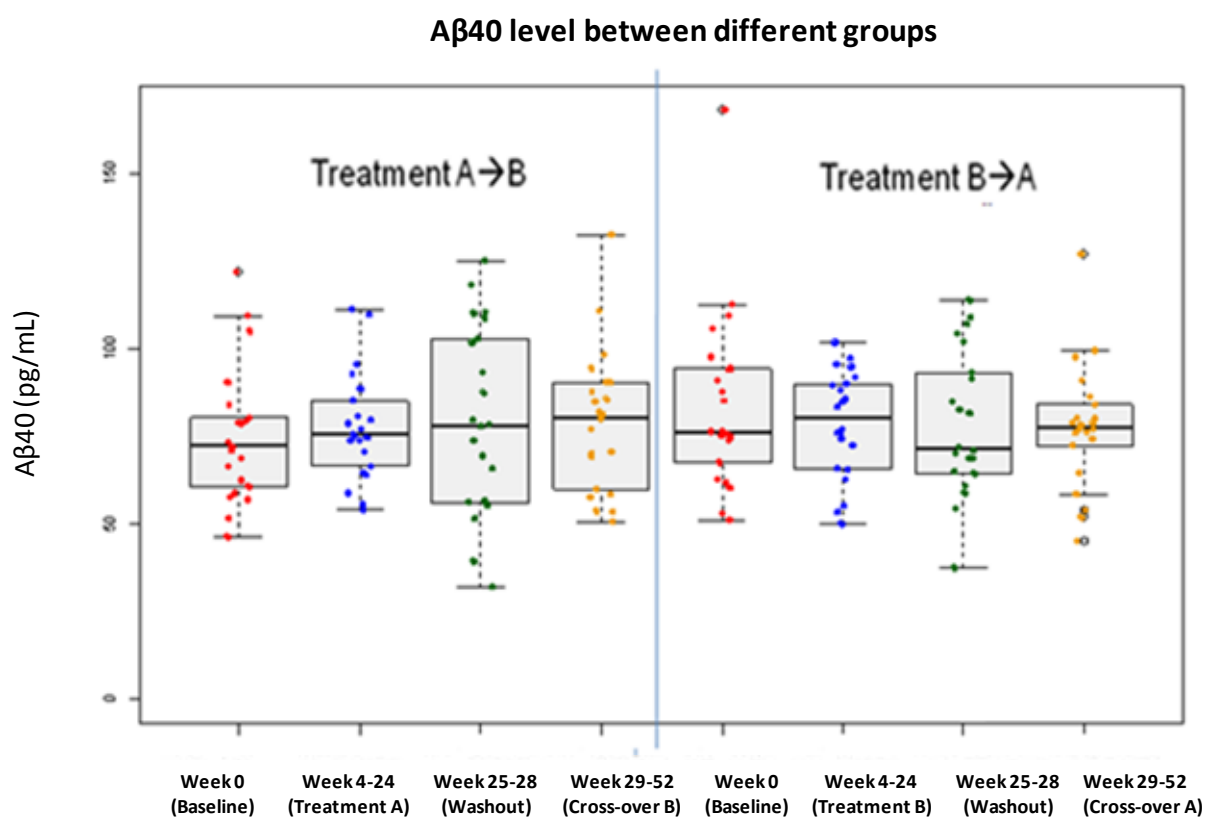
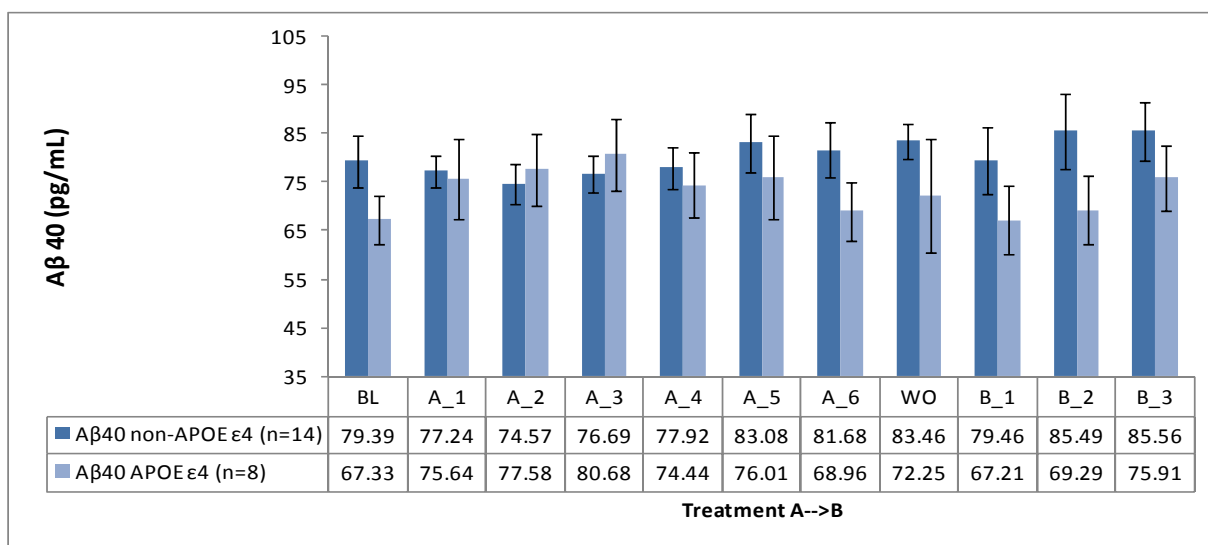


Figure 6.28 : Boxplots of average plasma A β 40 levels at each of the 4 stages of treatment A \rightarrow B and treatment B \rightarrow A. : Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in pg/mL and presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the Cross-over (second treatment) stages are the averages from 3 visits. There were no differences observed between any of the stages of either treatment arm.

(A)



(B)

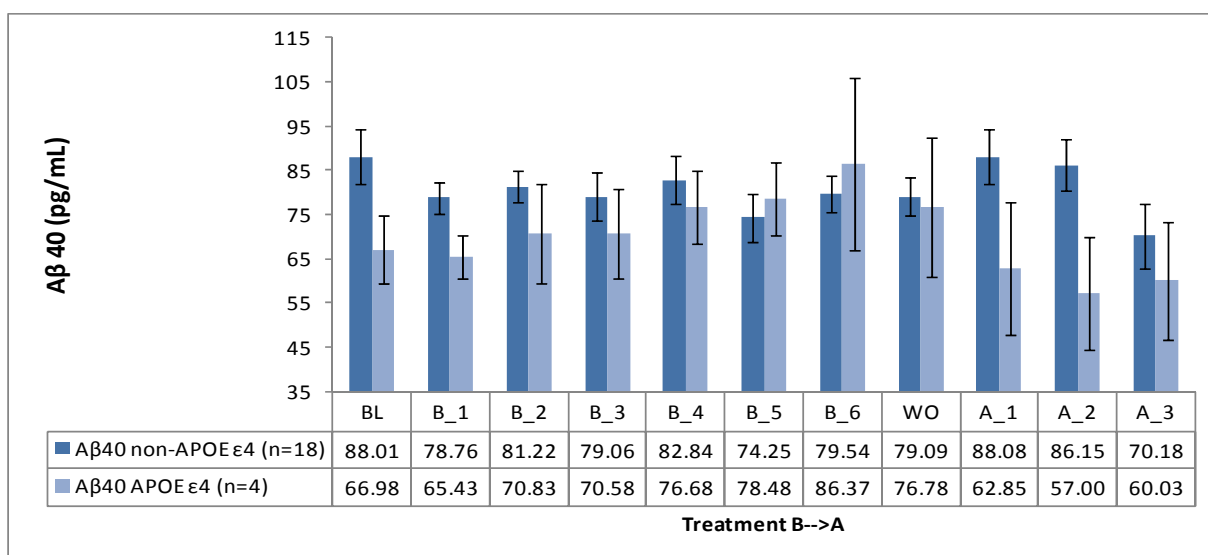


Figure 6.29 : Comparison of plasma Aβ40 levels in the non-APOE ε4 and APOE ε4 carriers at each of the 11 individual visits of treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in pg/mL underneath the bars are the mean of each group. No significant differences were observed between non-APOE ε4 and APOE ε4 participants. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

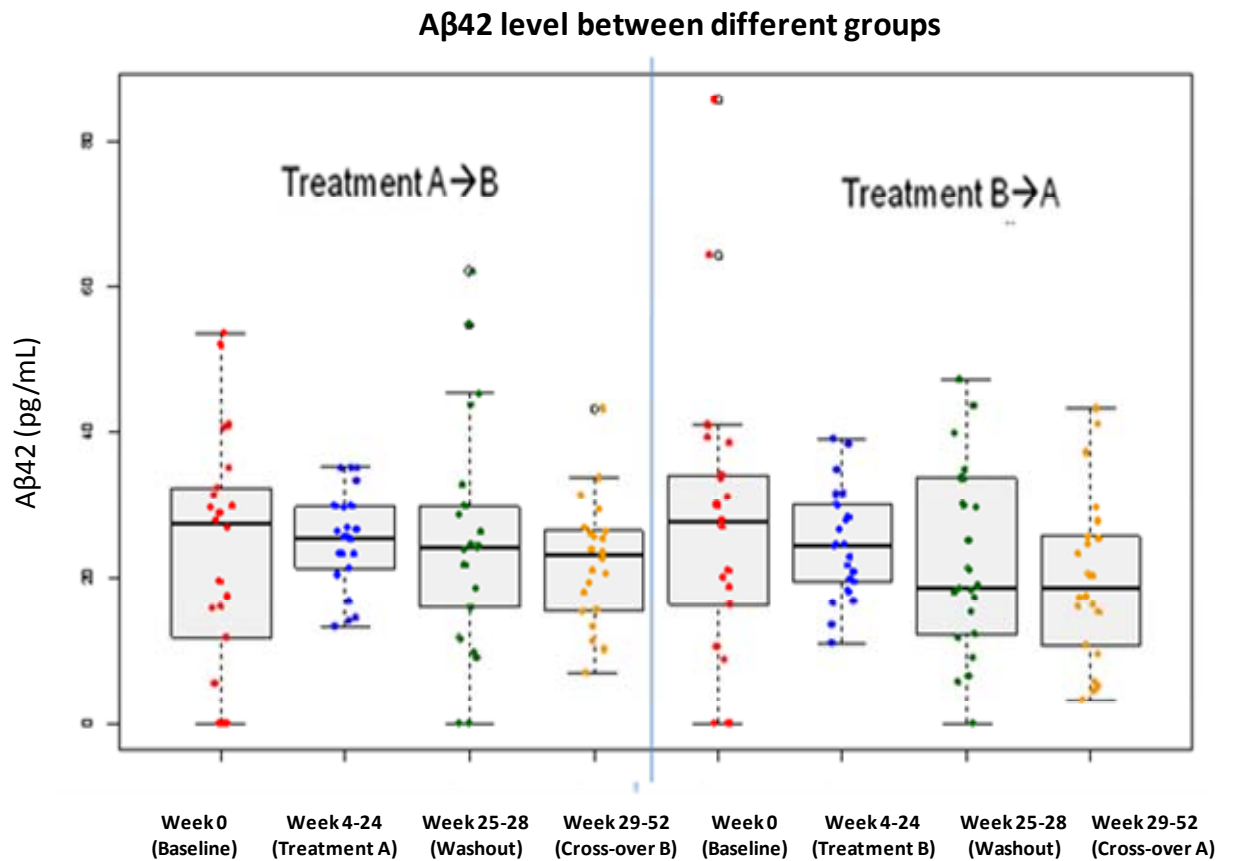
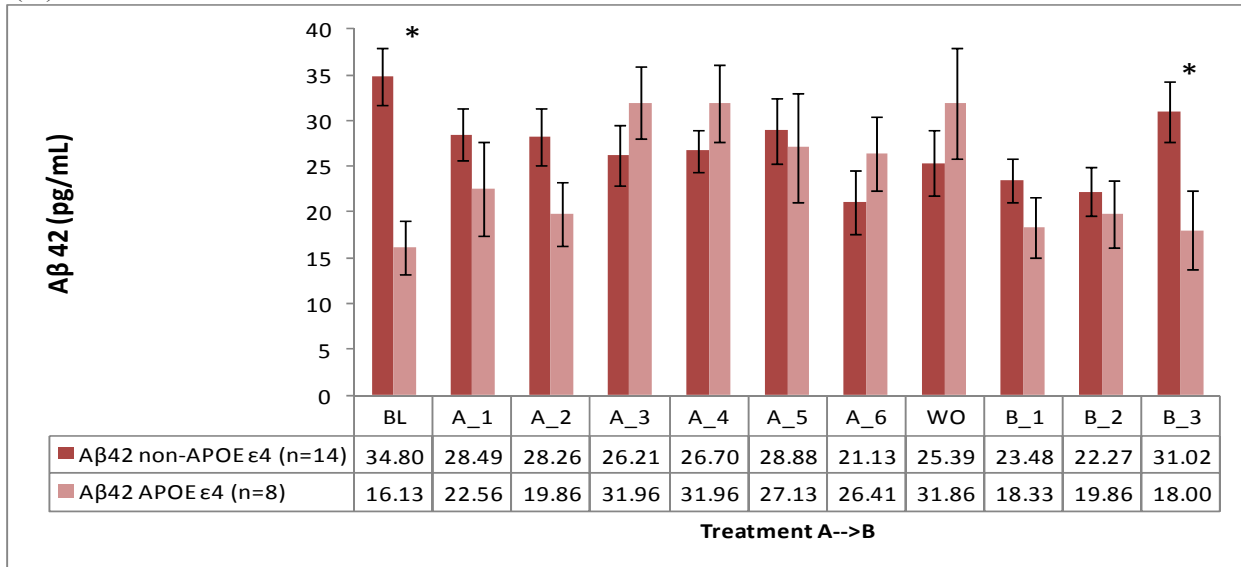


Figure 6.30 : Boxplots of average plasma A β 42 levels for each of the 4 stages of treatment A \rightarrow B and treatment B \rightarrow A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in pg/mL and presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the Cross-over (second treatment) stages are the average results from 3 visits. There were no differences observed between any of the stages of either treatment arm.

(A)



(B)

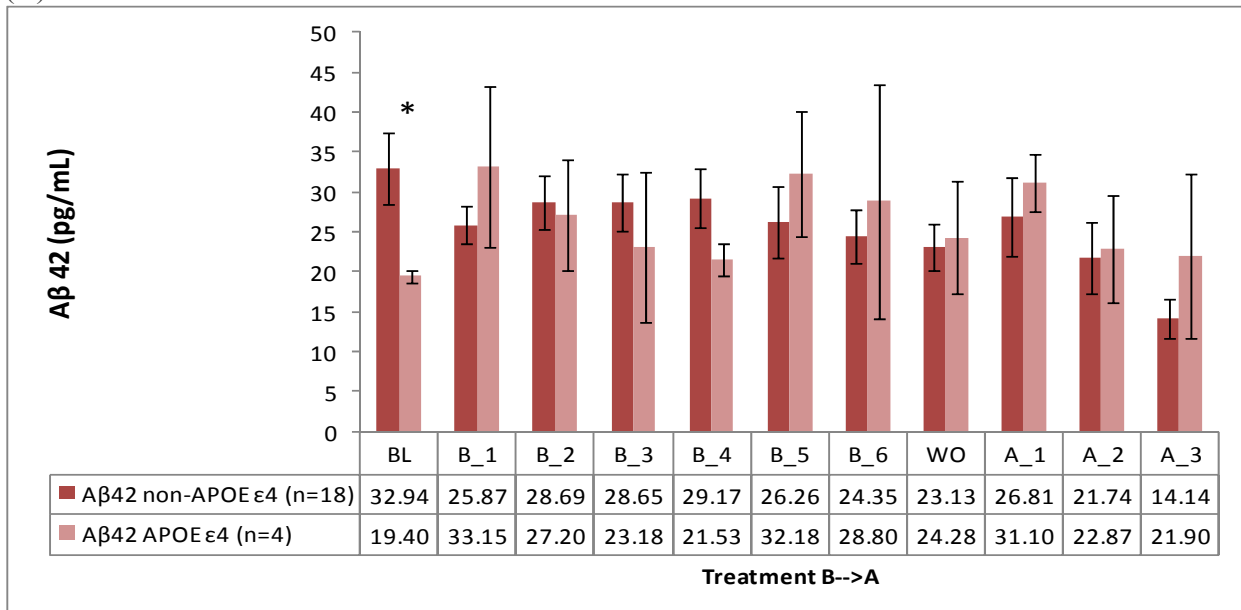


Figure 6.31 : Comparison of plasma Aβ42 levels in non-APOE ε4 and APOE ε4 carriers at each of the 11 visits of treatment A→B (A) and treatment B→A (B). The bars represent the mean ± standard deviation. Values in pg/mL underneath the bars are the mean of each group. Significant differences were observed between the APOE ε4 and non-APOE ε4 subjects at baseline of both arm and the end of the treatment in treatment arm A→B only (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22)

Plasma A β levels, including A β 40 as well as A β 42 were rarely found to be significantly different between any of the treatment stages. However, from the Figure 6.31 above, there seemed to be a trend towards decreasing A β 42 levels following testosterone treatment in the non-APOE ϵ 4 subjects of both treatment groups. In treatment A \rightarrow B, A1 to A6 show lower testosterone levels compared to baseline, however this doesn't return to baseline levels until end of placebo (cross-over) treatment. In Treatment B \rightarrow A, testosterone treatment is in the A1 to A3 time points, and a trend towards decreasing A β 42 levels can be seen in the non-APOE ϵ 4 subjects during the testosterone treatment period.

6.4.4. Effects of testosterone on plasma insulin levels

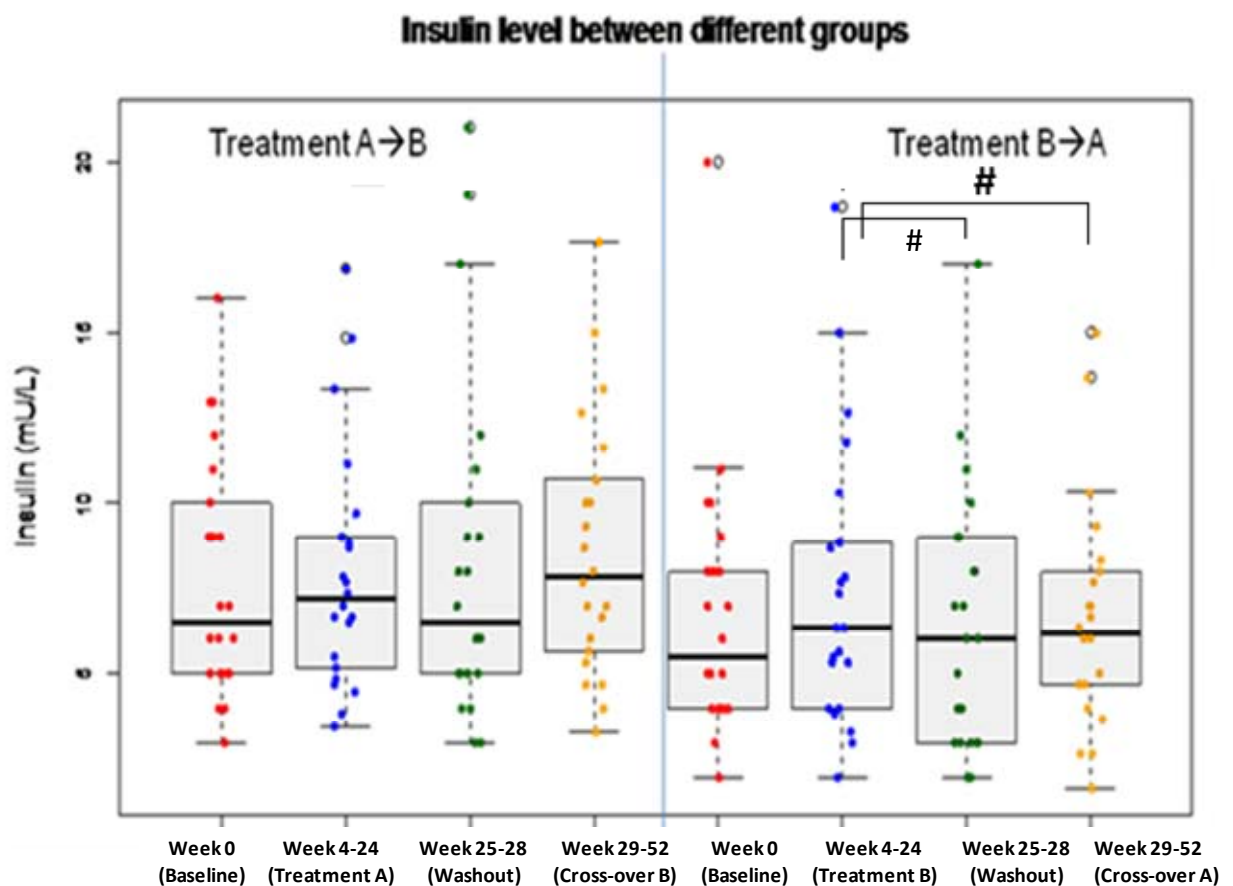
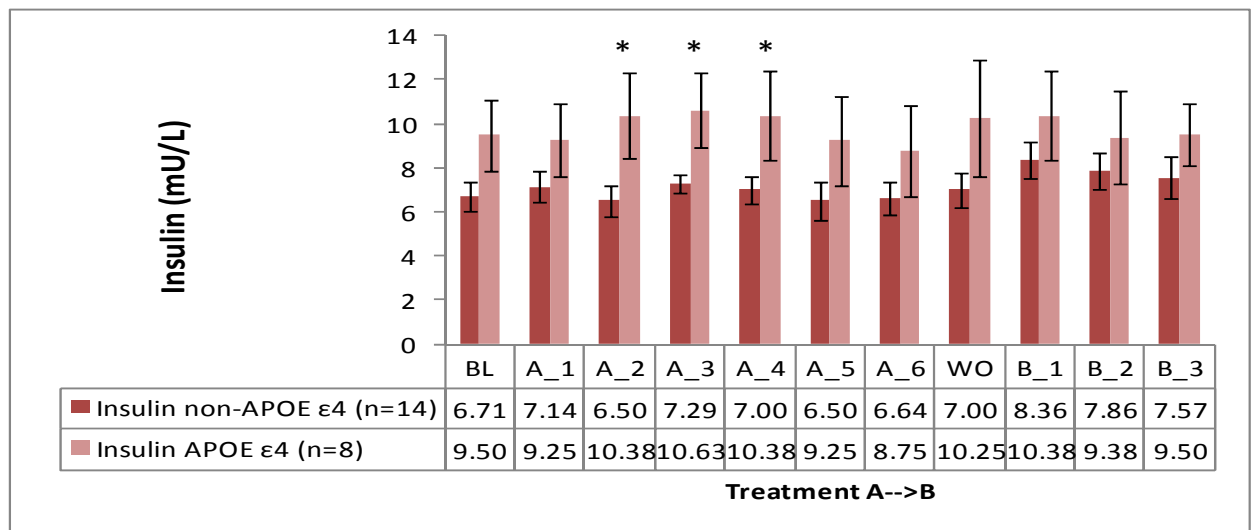


Figure 6.32 : Boxplots of insulin levels at each of the 4 stages of treatment A→B and treatment B→A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in mU/L and are presented as median \pm quartiles. The values in the first treatment stages are the averages of blood sample measurements from 6 visits and values in the Cross-over (second treatment) stages are the average results from 3 visits. Insulin levels were significantly increased in Treatment B compared to Cross-over A in treatment arm B→A. # $p<0.05$ but clinical relevance is questionable as no changes were seen when compared to baseline in the same treatment arm B to A.

(A)



(B)

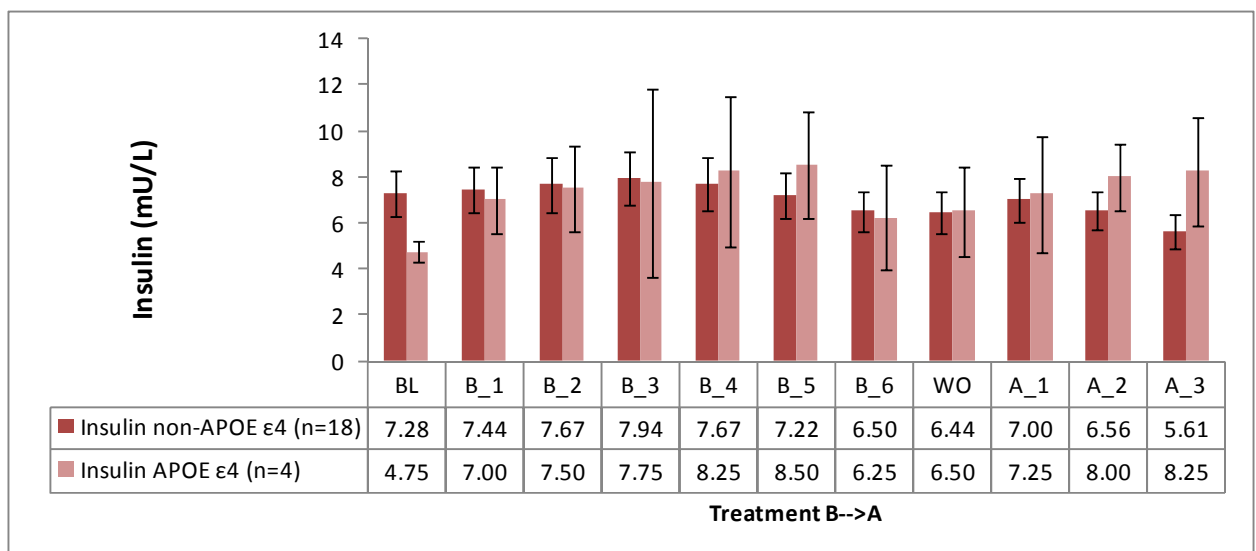


Figure 6.33 : Comparison of plasma insulin levels in non-APOE ε4 and APOE ε4 carriers at each of the individual 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in mU/L underneath the bars are the mean of each group. Significant differences were observed between the non-APOE ε4 and APOE ε4 groups of treatment arm A→B (* p <0.05), participants with APOE ε4 alleles had higher insulin levels. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22)

6.4.5 Effect of testosterone on Prostate Specific Antigen (PSA) levels in the plasma, assessed to investigate testosterone treatment safety

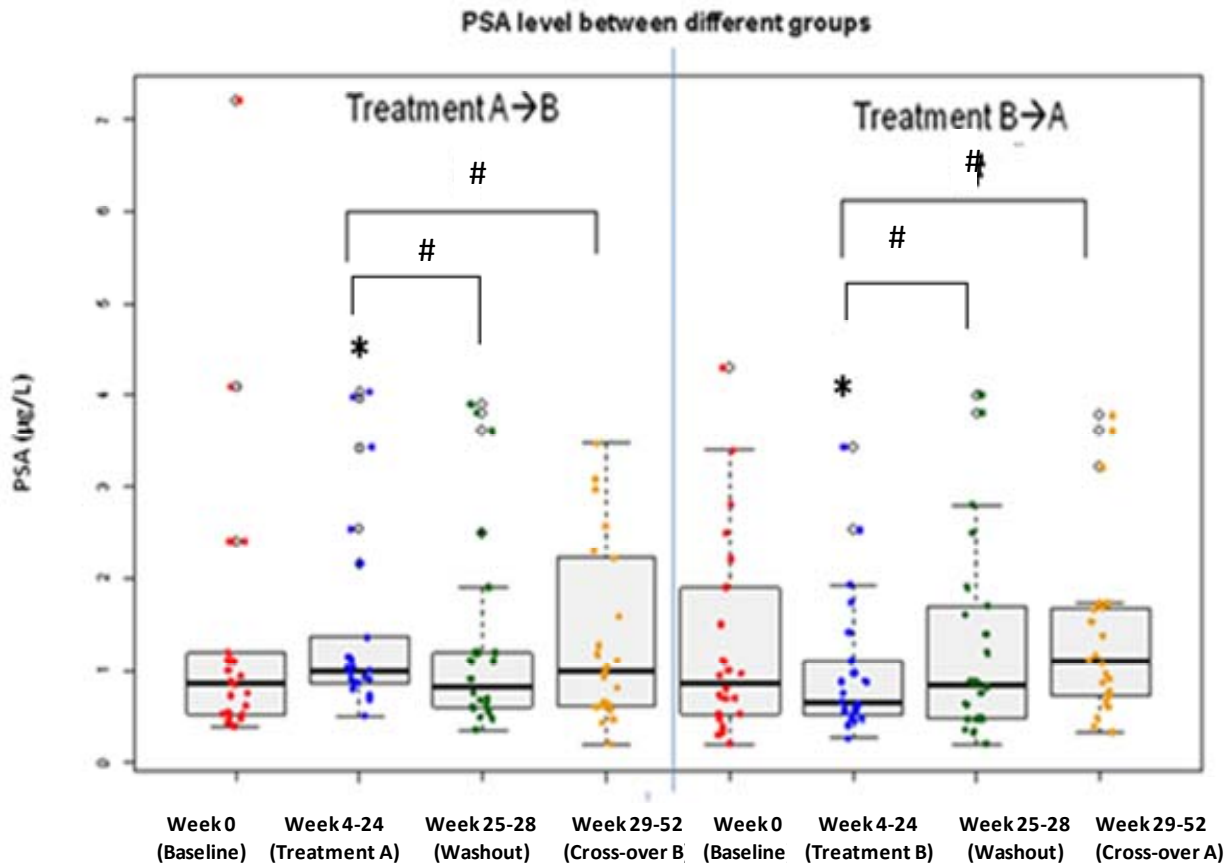
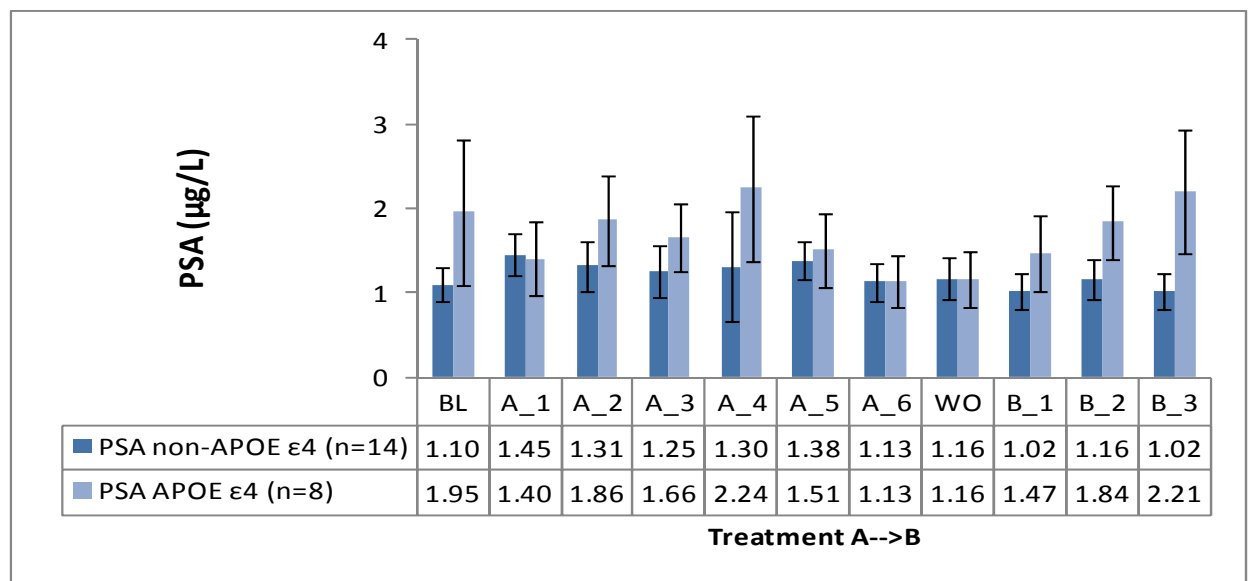


Figure 6.34 : Boxplots of PSA levels at each of the 4 stages of treatment A→B and treatment B→A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Data are in µg/L and presented as median ± quartiles. The values in the first Treatment stages are the averages of blood sample measurements from 6 visits and values in the Cross-over (second treatment) stages are the average results from 3 visits. Significant differences were observed in Treatment A and Treatment B compared to baseline (*p<0.05). There were also significant differences observed between the testosterone and placebo groups of both arms: the testosterone treatment groups had higher PSA levels when compared to placebo (#p<0.05).

(A)



(B)

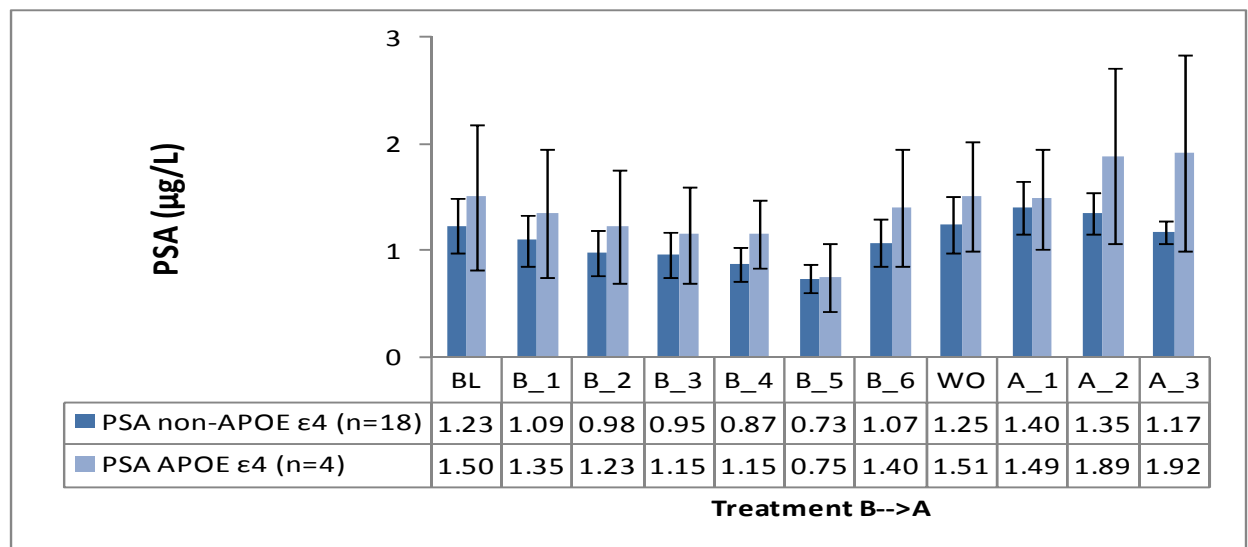


Figure 6.35 : Comparison of Prostate Specific Antigen (PSA) levels in non-APOE ε4 and APOE ε4 carriers at each of the 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in µg/L underneath the bars are the mean of each group. Although not significant, participants with APOE ε4 alleles had higher PSA levels compared to the non-APOE ε4 participants. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

6.4.6 Effects of testosterone on Red Blood Cells and Hemoglobin levels in the plasma

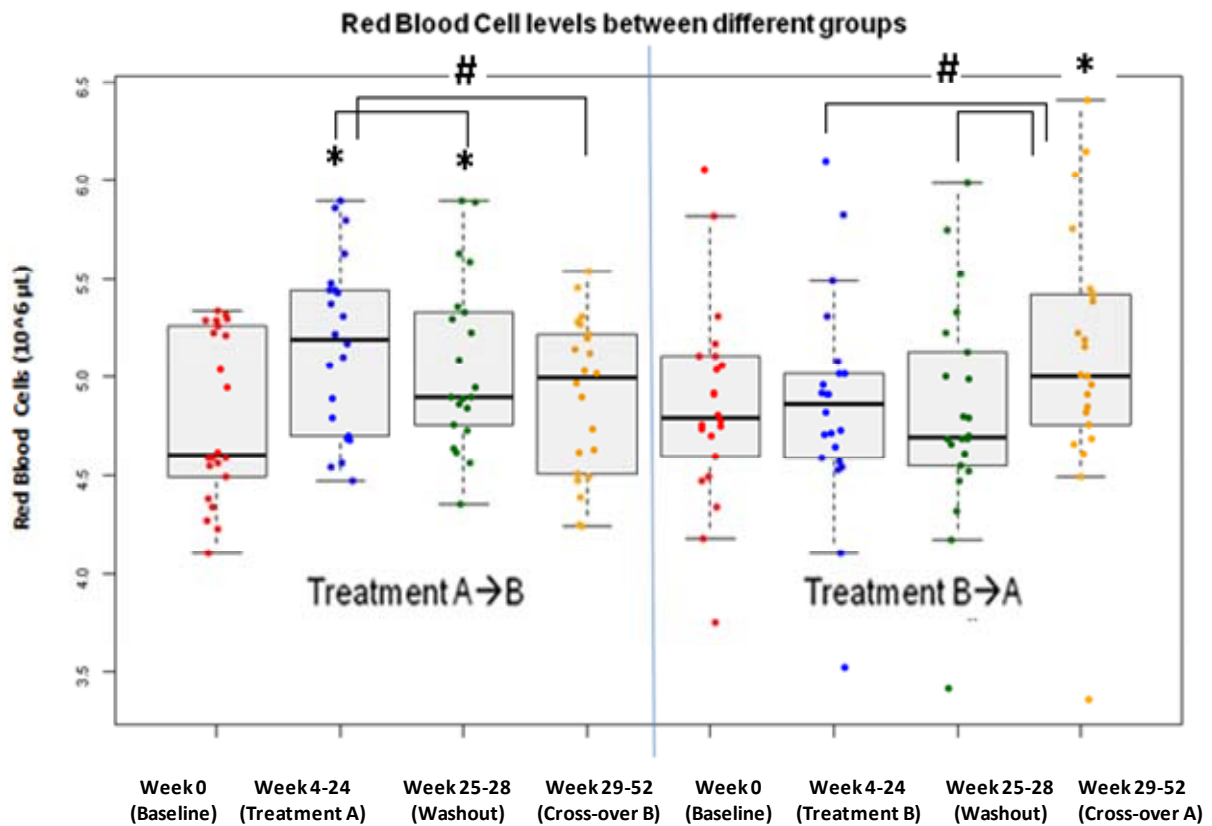
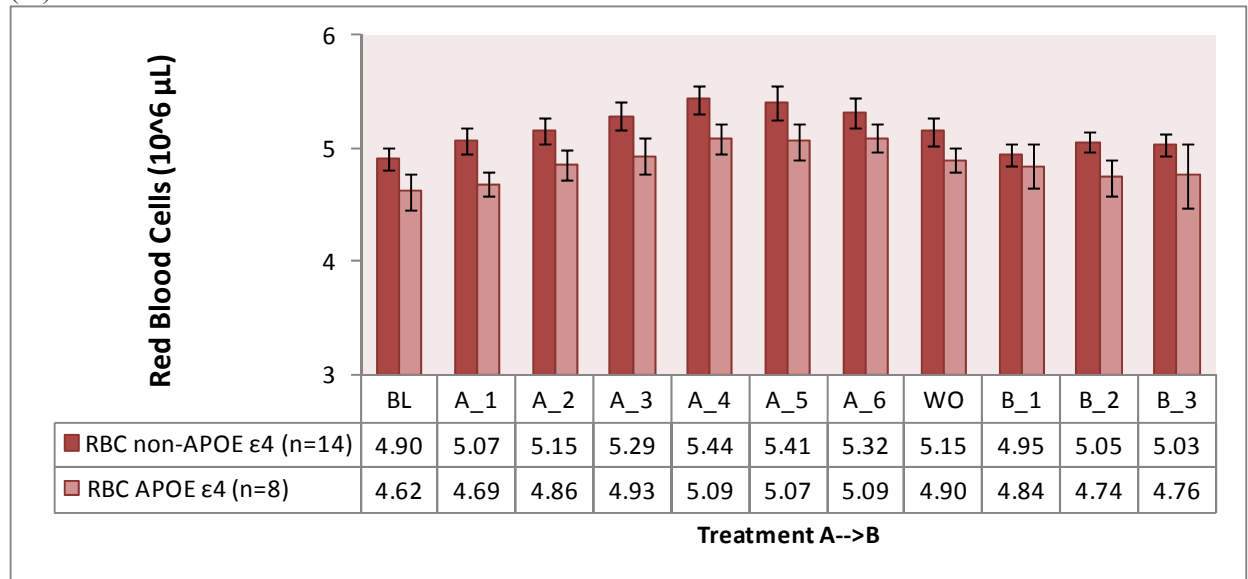


Figure 6.36 : Boxplots of Red Blood Cell counts (RBC) at each of the 4 stages of treatment A→B and treatment B→A: Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Values are in 10^6 cells/ μL and are presented as mean \pm quartiles. There were significant increases in RBC levels following testosterone treatment compared to baseline levels in both treatment arms (* $p < 0.05$), as well as compared to other groups (# $p < 0.05$).

(A)



(B)

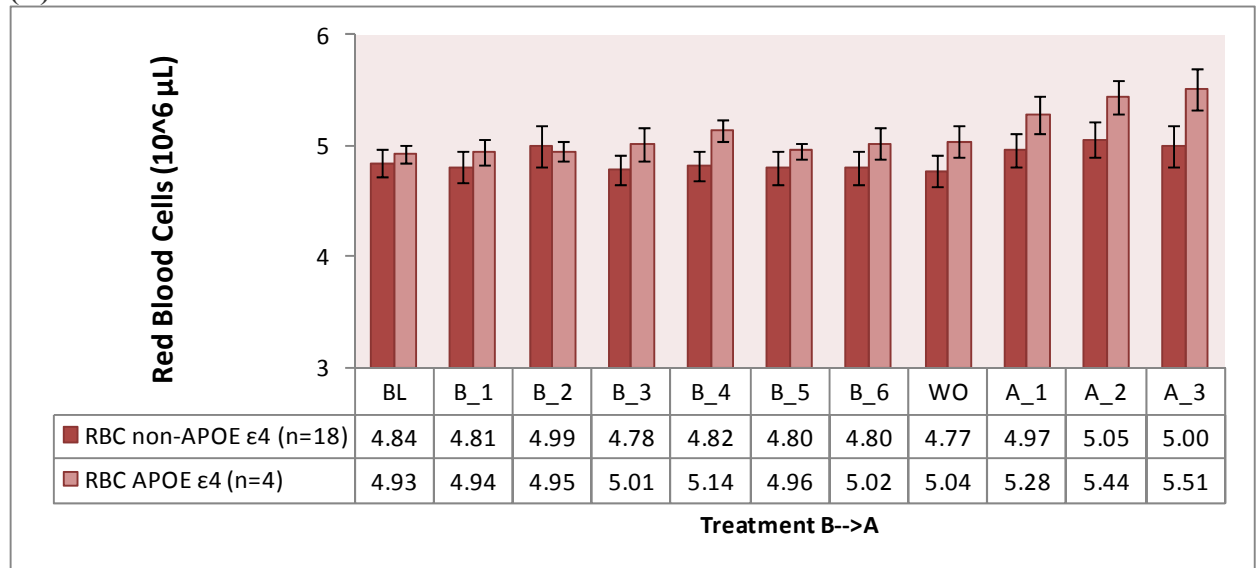
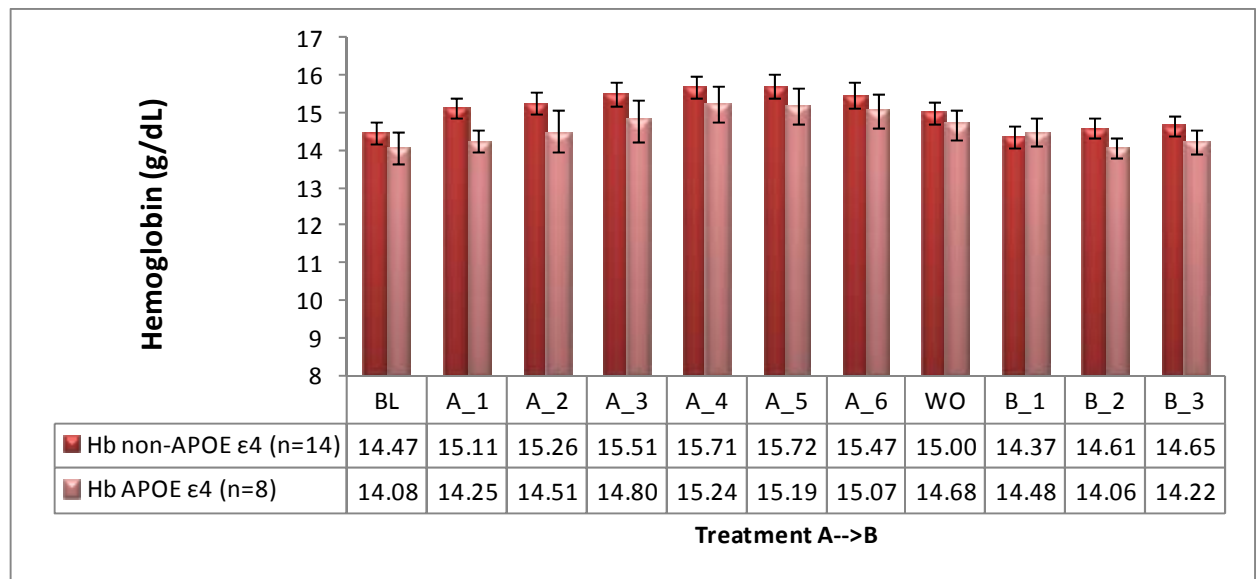


Figure 6.37 : Comparison of Red Blood Cell (RBC) levels in non-APOE ε4 and APOE ε4 carriers at each of the individual 11 visits of treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in 10^6 cells/ μ L underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ε4 and APOE ε4 groups of the treatment arms. BL= Baseline, A_1 to A_6 & B_1 to B_6 = Treatment period, WO=Wash-out, B_1 to B_3 & A_1 to A_3 = Cross-over period. (n=22).

(A)



(B)

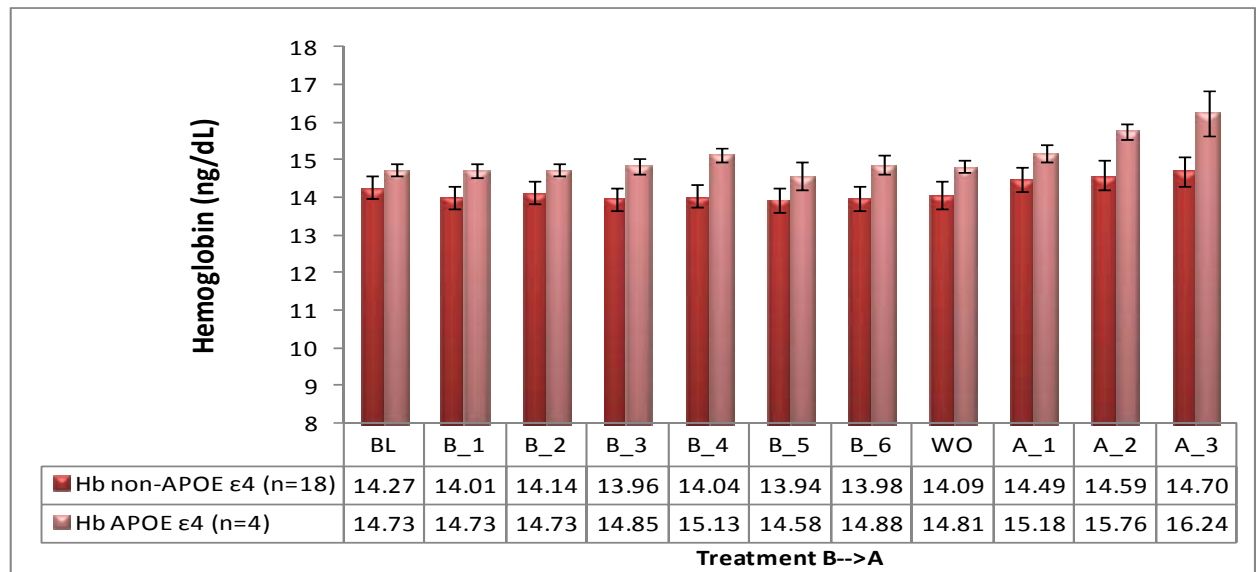


Figure 6.39: Comparison of hemoglobin (Hb) levels in non-APOE ε4 and APOE ε4 carriers at the individual 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in g/dL underneath the bars are the mean of each group. There were no significant differences observed between the non-APOE ε4 and APOE ε4 groups of either arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

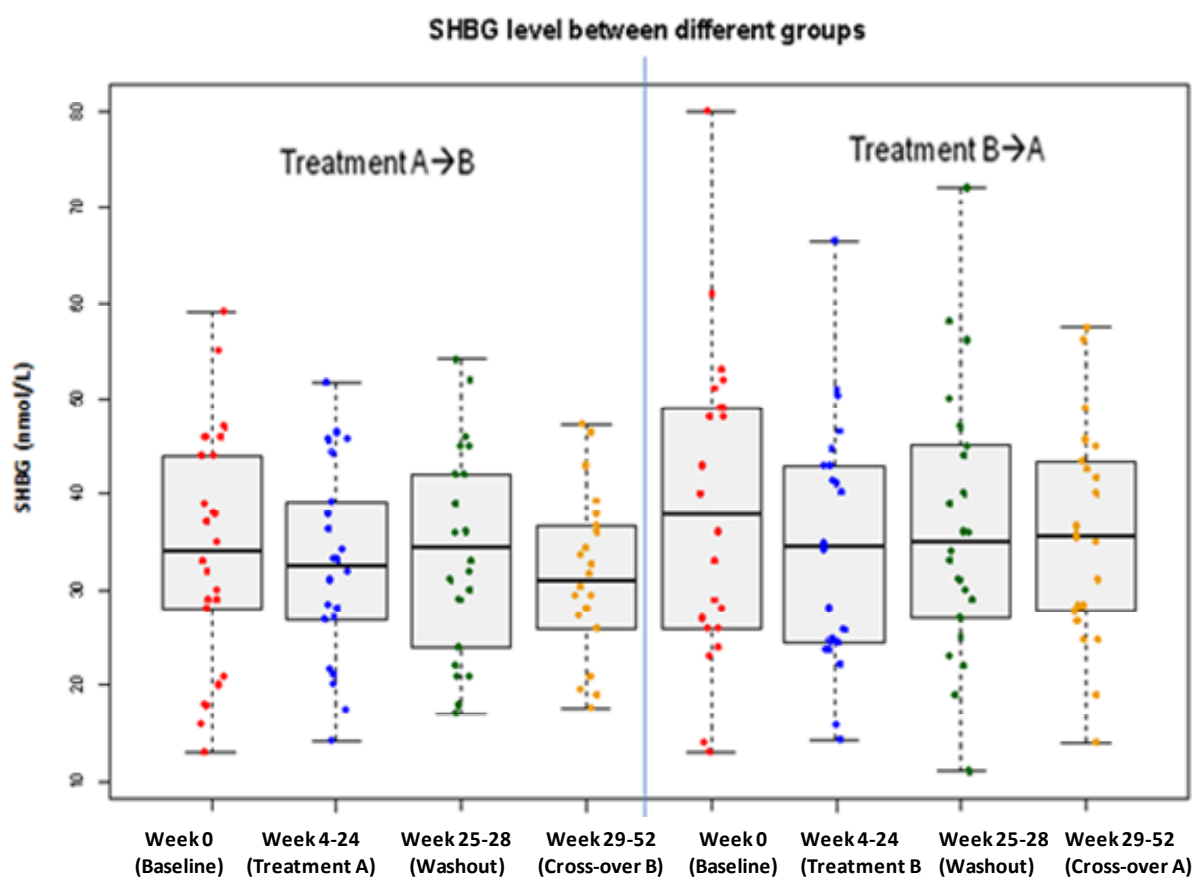


Figure 6.40 : Boxplots of serum Sex Hormone Binding Globulin (SHBG) levels at the 4 different stages of treatment A→B (A) and treatment B→A (B). Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Values are in nmol/L and are presented as mean \pm quartiles. No significant differences were observed between any of the stages of either treatment arm.

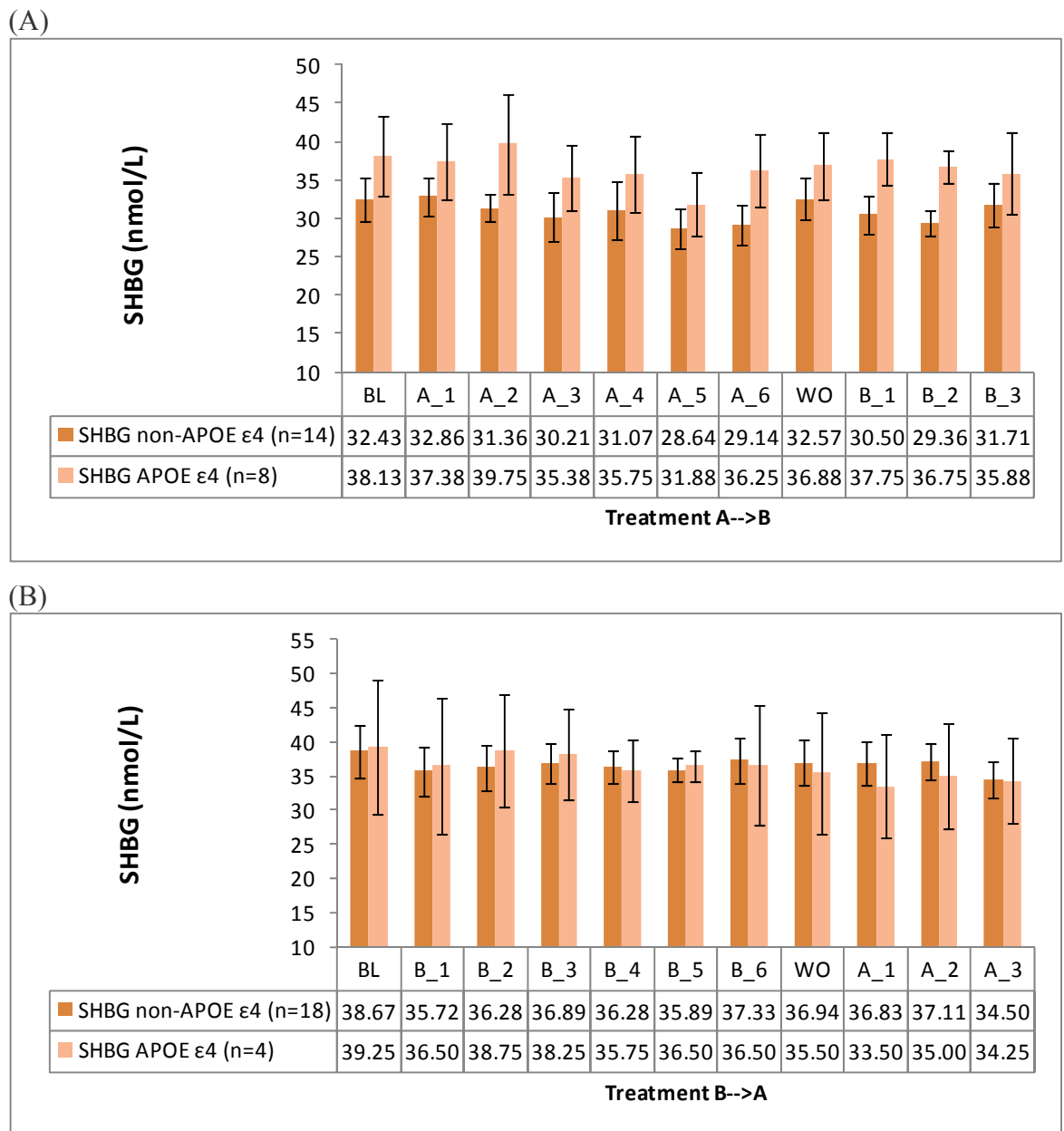


Figure 6.41: Comparison of hemoglobin (Hb) levels in non-APOE ε4 and APOE ε4 carriers at the individual 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in nmol/L underneath the bars are the mean of each group. There were no significant differences observed between the non-APOE ε4 and APOE ε4 subjects of either treatment arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

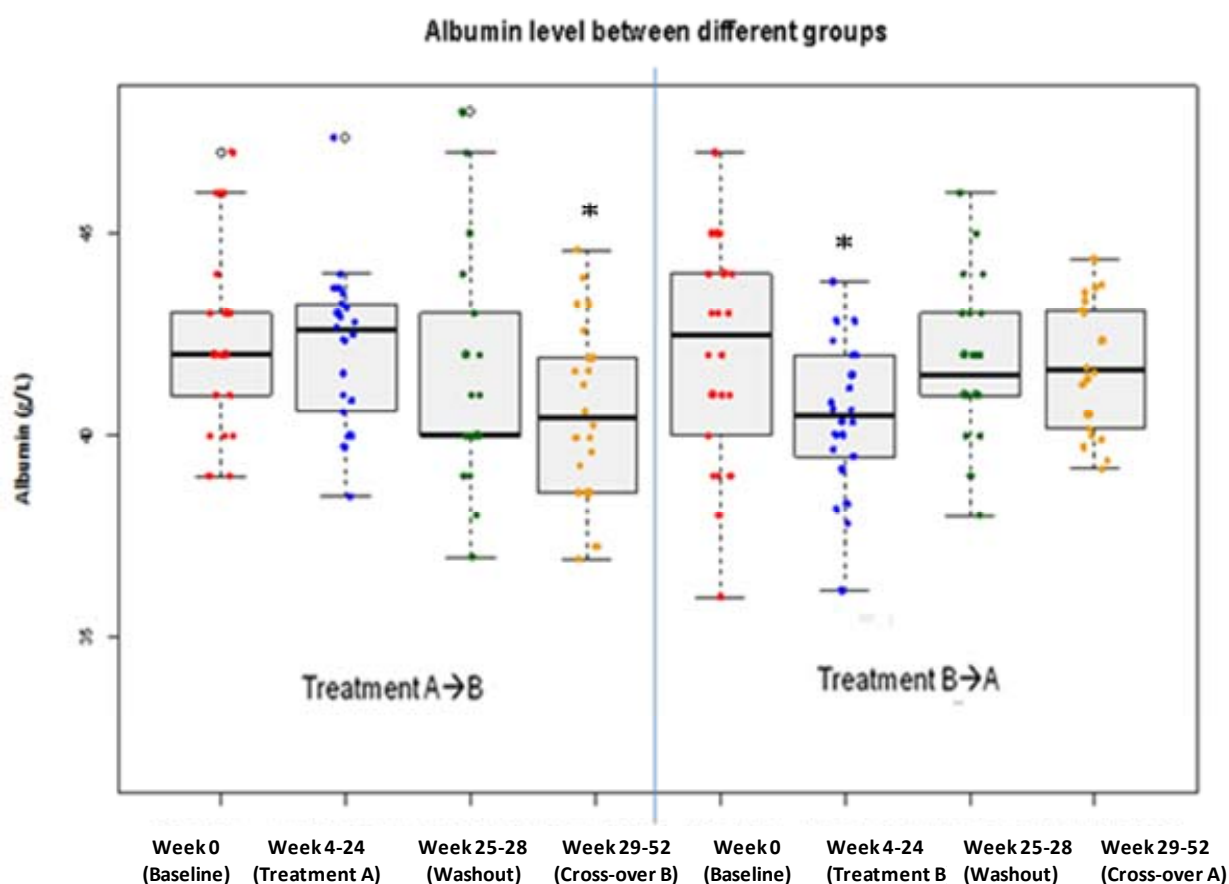
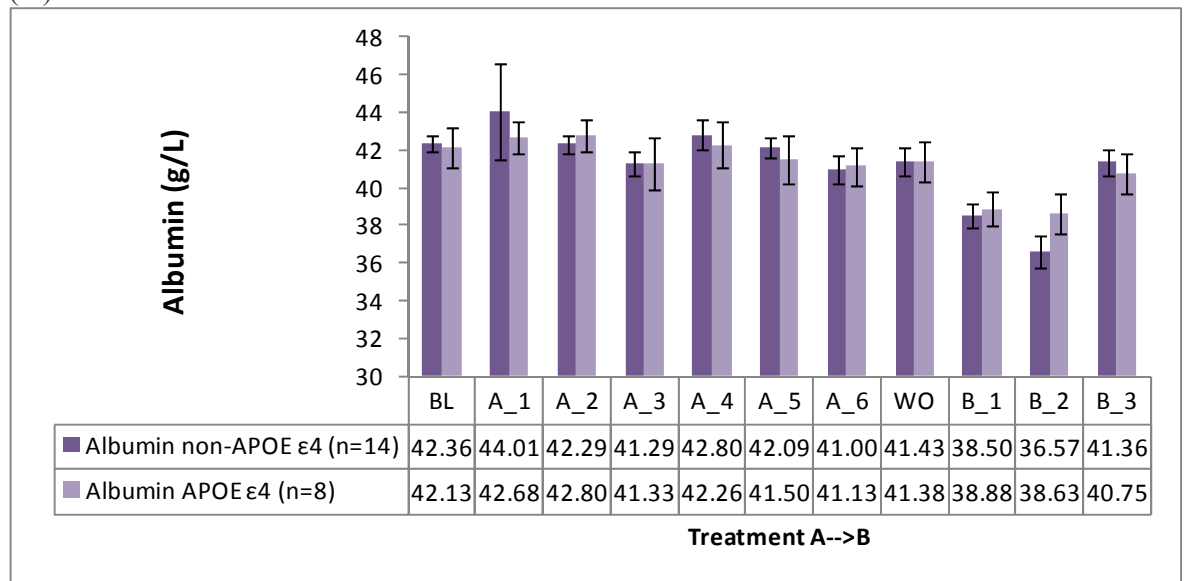


Figure 6.42: Boxplots of serum Albumin levels at the 4 stages of treatment A→B (A) and treatment B→A (B). Week 0 (Baseline), Week 4-24 (Treatment), Week 25-28 (Washout), and Week 29-52 (Cross-over). Values are in g/L and are presented as mean \pm quartiles. Compared to baseline levels, albumin levels were significantly lower during the placebo treatment, in both treatment arms. (* $p < 0.05$).

(A)



(B)

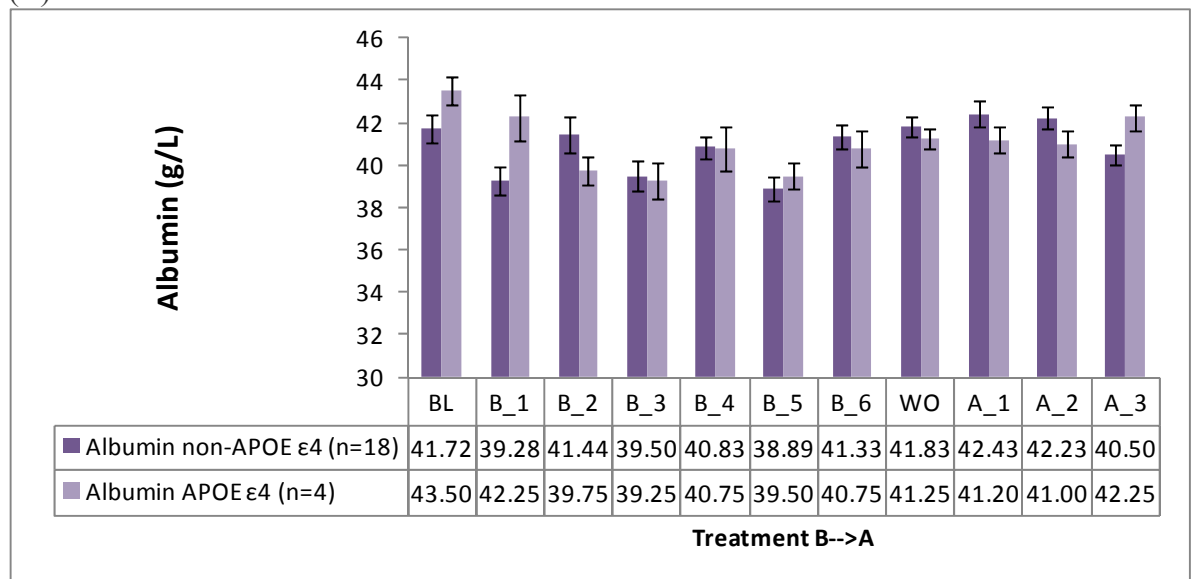
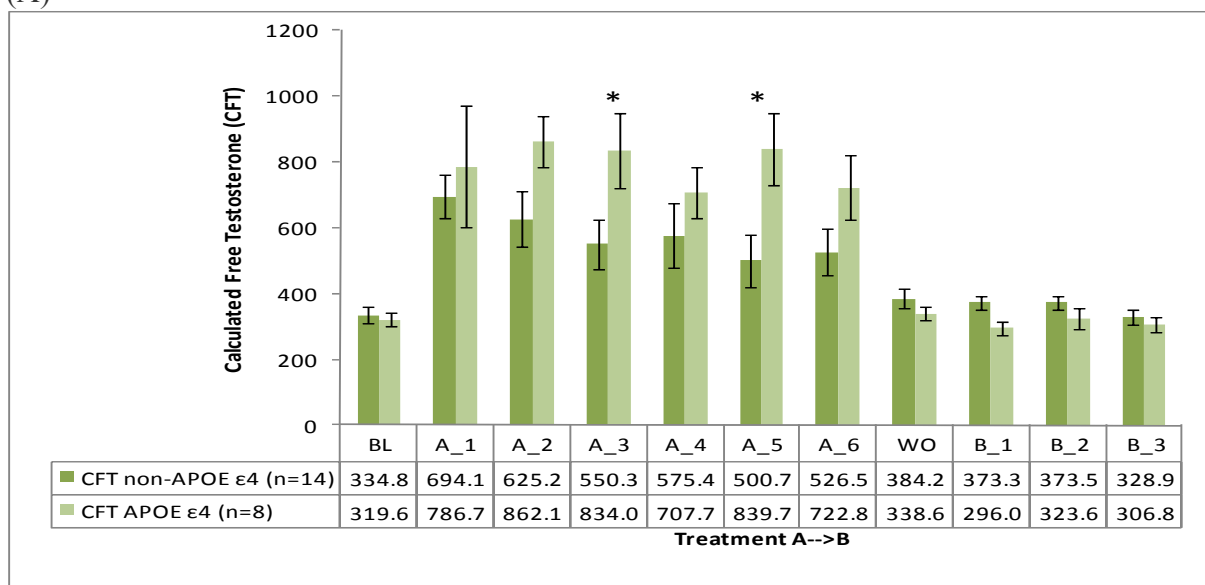


Figure 6.43 : Comparison of albumin levels in non-APOE ε4 and APOE ε4 carriers at the 11 individual visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in g/L underneath the bars are the mean of each group. No significant differences were observed between the non-APOE ε4 and APOE ε4 groups of either treatment arm. BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period. (n=22).

(A)



(B)

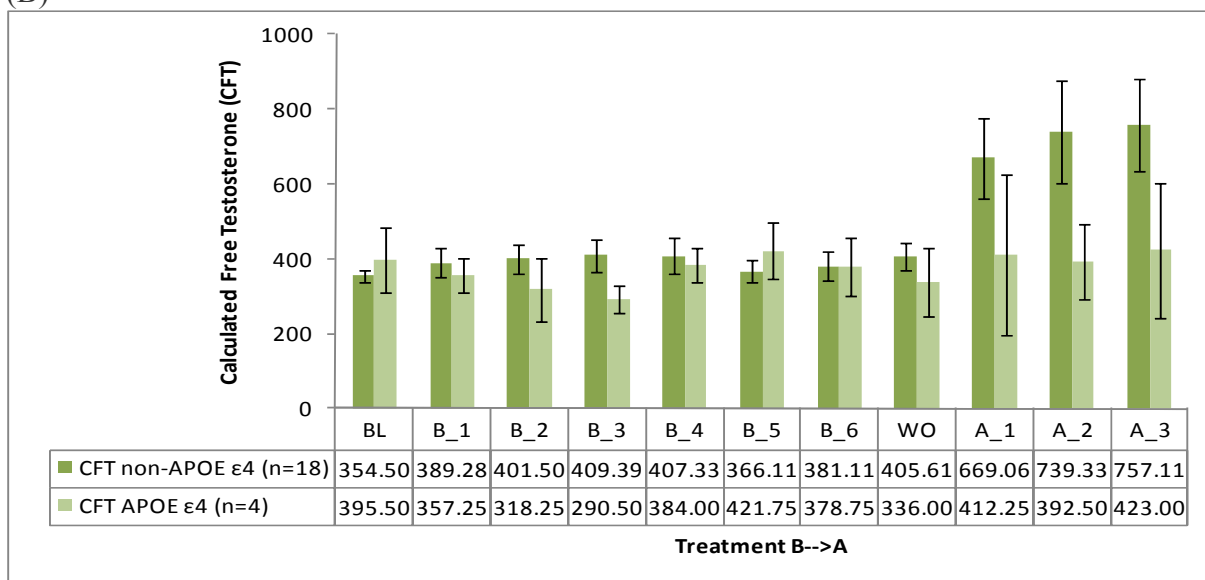


Figure 6.44 : Comparison of Calculated Free Testosterone levels in APOE ε4 and non-APOE ε4 carriers (n=22) at 11 visits in treatment A→B (A) and treatment B→A (B). The bars represent the mean \pm standard deviation. Values in ng/dL underneath the bars are the mean of each group. Significant differences were observed between non-APOE ε4 and APOE ε4 group in treatment arm A→B (*p<0.05). BL= Baseline, A_1 to A_6 (graph A) & B_1 to B_6 (graph B) = First Treatment period, WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (graph B) = Second (Cross-over) treatment period.

6.5 Discussion

Our knowledge and understanding of testosterone replacement therapy in aging males has greatly improved over the last 15-20 years, however data on many of its effects remain inconclusive. There is a growing need for clinical studies that can help us improve our understanding of the long-term effects of testosterone treatment.

The study design in this trial involved the treatment of subjective memory complainers for a period of nearly 6 months. This length of time was necessary to differentiate true benefits of therapy from placebo effects. Moreover, in this double blind, randomized, and placebo-controlled trial, all participants underwent both testosterone treatments and placebo treatments within the trial's one year duration, thus all participants served as their own "controls". Part of the strength of this study is due to this design, as it reduced the problems of patient to patient variability when comparing the placebo to the testosterone effect. Another strength of the study came from the fact that both potential orders of treatment were tested – testosterone followed by placebo, as well as the reverse. From the point of view of the participants, their role was the same for a whole year – for all stages a cream was to be applied daily.

In the initial screening of elderly men who presented with signs and symptoms of testosterone deficiency, we measured total testosterone levels. The conventional view is that total testosterone is the test of choice, it also has the advantage of being one of the most widely available and the gold standard of the testosterone assays. Total testosterone levels were also used to monitor the efficacy of testosterone treatment. Total plasma testosterone represents testosterone that is tightly bound to sex hormone binding protein (SHBG, 44%) as well as that weakly associated with albumin (54%), with the remaining 2% free (and able to pass the blood brain barrier) (Bates *et al.*, 2005). With aging, the rate of decrease in total testosterone level is on average, approximately 110 ng/dL (~3.8 nmol/L) per decade (Morley *et al.*, 1997). The most commonly used test is early morning serum total testosterone level. If it is less than 300 ng/dL (~ 10.4 nmol/L) then the patient should be considered testosterone deficient (Basaria and Dobs, 2001). However, the threshold of testosterone levels varies for various symptoms and among individuals. Most androgen deficiency symptoms will occur in men who have

testosterone levels that correspond to or are below the lower limit of the normal range for young men, which is 10.4 nmol/L (Wu *et al.*, 2010; Orwoll *et al.*, 2010; Mulligan *et al.*, 2006; Araujo *et al.*, 2007; Kelleher *et al.*, 2004). The mean of the total testosterone levels from the 44 participants in our study was slightly above the borderline for testosterone deficient men, since the determination of the borderline was based on the normal testosterone levels found in Indonesian people in Siloam Hospitals, using VIDAS Testosterone Immunoassay kit (Bio-Merieux). After the samples being analyzed with gas chromatography-mass spectrometry (GC-MS), the level of total testosterone levels at baseline was above 300 ng/dL. Therefore, the limitation in this study is the screening measurement methods of total testosterone. The therapy was aimed to raise serum testosterone levels to that seen in the mid-normal range, which for these Indonesian people was 600-1000 ng/dL, based on the laboratory reference range using immunoassays. In addition, calculated free testosterone was also measured in this experiment, and showed similar results to those observed with total testosterone levels following testosterone treatment (Fig 6.44).

6.5.1 Effects of testosterone administration on plasma total testosterone levels and its metabolites, DHT and estradiol

The day to day variation in testosterone levels may be considerable, therefore repeated testosterone measurements at 4 week intervals in weeks 4-24 (first treatment period) and at 8 week intervals in weeks 28-52 (second treatment period) were undertaken. From the total testosterone level results (Figs 6.2 & 6.3) it is easy to see that treatment A is testosterone whilst treatment B is placebo. Testosterone levels were significantly higher following testosterone treatment in both treatment groups. Within four weeks post-testosterone treatment, referred to as the washout period, testosterone levels were back to that seen at baseline which indicated no carry-over effects. Individual levels of testosterone varied in response to testosterone treatment (Fig 6.8 & 6.9; Appendices Table 1 & 2). This may have been due to the androgen receptor polymorphisms (CAG repeats) conferring differential individual sensitivity to testosterone or due to the possibility that inadvertently different amounts of testosterone may have been applied for some individuals. Previous studies have shown that the length of the CAG repeats in

exon 1 of the androgen receptor determines the transactivation activity of the receptor, in which shorter CAG repeats are associated with higher androgenicity and longer CAG repeats with lower androgenicity in healthy (Zitzmann *et al.*, 2001; Zitzmann *et al.*, 2003a) as well as in testosterone deficient men (Zitzmann *et al.*, 2003b). Furthermore, the number of androgen receptor CAG repeats is negatively correlated with AR sensitivity and positively correlated with body fat, insulin levels, and leptin in healthy men (Stanworth *et al.*, 2008).

The mechanisms of action of testosterone on target organs are relatively complex since effects may be mediated directly via testosterone or indirectly via its metabolic products, dihydrotestosterone (DHT) and estradiol. Furthermore, testosterone only has a short half-life of approximately 12 minutes in the blood (Carruthers, 2004). In the current study, the testosterone cream resulted in elevated serum DHT levels with no carry-over effect, most likely as a result of the high concentrations of 5- α reductase in the scrotum. The DHT produced would have been available to bind to androgen receptors (Fig 6.4 & 6.5). These results are in agreement with those of Cunningham *et al.*, 1989, where it was found that DHT is elevated following testosterone treatment using a transdermal cream. The results in Figs 6.6 and 6.7 show that elevating testosterone levels resulted in a relatively greater increase in DHT, as observed by the increased ratios in these figures. These results indicated that the testosterone has been converted mostly to DHT, which is consistent with application of the cream-based hormone to the scrotum.

The transport of testosterone and its metabolites into various tissues of the body is dependent on their binding to the transport proteins, their rate of dissociation, their membrane permeability and their capillary transit time through an organ (Pardridge, W.M, 1981). Testosterone is known to affect body composition in men, for example testosterone reduces abdominal fat and increases muscle mass. The quantity of fat, both subcutaneous as well as visceral, correlates with levels of both testosterone and DHT (Nielsen *et al.* 2007). DHT and testosterone levels also affect proliferation of fat cells - they block the transformation of pluripotent cells into adipocytes (Singh *et al.* 2003). It has also been demonstrated that unlike testosterone, DHT has a positive effect

on bone density (Ilangovan *et al.* 2009). These are all studies that support the notion that low testosterone and DHT are detrimental to health.

Determining whether testosterone or DHT levels are low or not, is in itself difficult – normally ratios of DHT/testosterone do not vary much past puberty, yet can be different depending on race. For example, although testosterone levels were found to be very similar in a large US study, higher DHT levels and lower DHT/testosterone ratios were found in black people when compared to white or Hispanic people. Geographical differences have also been found, with people from similar racial backgrounds having quite different DHT levels depending on country of residence (Duskova and Pospisilova, 2011). It is likely that diet and other health issues may play an important contributory role to these differences.

The conversion of testosterone to estradiol via the enzyme cytochrome P450 aromatase in men, unlike women, helps to maintain or modulate levels of serum estradiol as men age (Burger, 2000; Gray, 1991). Thus, in the presence of aromatase, testosterone can exert its effects on cell metabolism by influencing estrogen receptors as well as androgen receptors (Tietz, 1986). Our results show moderate changes in estradiol levels do occur following testosterone treatment (Fig 6.10 & 6.11).

However, some studies have observed an increase in estradiol levels with age, resulting from increased adiposity or increased body fat which would be responsible for an increase in levels of serum sex-hormone-binding globulin (SHBG) and enhanced peripheral aromatization of androgens (Basaria *et al.*, 2001). Estradiol should be in the mid- to lower-normal range, which is 10-82 pg/mL in fertile Asian men (Yamamoto *et al.*, 1995). If estradiol levels are at least one-third above the normal reference range, this excessive level of estradiol should be reduced. The results from this study showed that estradiol levels were still in the normal reference range in baseline and all of the treatment groups (Fig 6.10 & 6.11).

The normal production ratio of testosterone to estradiol is approximately 100:1 and the normal circulating ratio of testosterone to estradiol is approximately 300:1 (www.emedicine.medscape.com). Increased production or action of estradiol compared to testosterone will result in gynecomastia: benign enlargement of the male breast; an unusual side effect associated with testosterone replacement therapy. Rhoden and

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Morgentaler, 2004 have shown that 6 months of testosterone treatment can induce gynecomastia in men with history of gynecomastia. Therefore, in these studies the ratio of testosterone to estradiol was also calculated to monitor the risk of gynecomastia. Findings from this current study revealed an almost 2-fold increased ratio of circulating testosterone to estradiol in the first 4 weeks following testosterone treatment in both arms, which subsequently decreased until the end of the treatment (Fig 6.12). These results indicate that increased testosterone levels were more prominent than the increased estradiol levels which indicates that risk of gynecomastia was minimal following this testosterone treatment.

From Figures 6.14 and 6.15, it can be seen that testosterone, DHT, and estradiol had inhibitory effects on LH levels in plasma. Once the participants stopped using the testosterone cream, the LH levels rose back to levels seen in the baseline readings which indicated no carry-over or long-term effects. Thus the normal feedback loops and enzymatic conversions of testosterone were occurring during the treatment times. In men, both testosterone and DHT exert their effects on LH mainly at the hypothalamic level, by decreasing the frequency of LH pulses, whilst estradiol reduces the amplitude of LH at the pituitary level (Carruthers, 2004). Among testosterone deficient men, LH has been shown to be suppressed (reduced) down to levels in the eugonadal range following injectable and implantable testosterone (Fennel *et al.*, 2010; Conway *et al.*, 1988).

Apolipoprotein E (ApoE), a major carrier of cholesterol in the central nervous system is produced from the APOE gene, which exists mainly as three different alleles in humans – APOE ϵ 2, ϵ 3 and ϵ 4. The possession of one or more APOE ϵ 4 alleles is the strongest known risk factor for sporadic or late onset AD. Participant APOE allele status was also determined in this study to try to evaluate any effect apoE protein isoform differences may have on testosterone treatment. Hogervorst *et al.*, 2002 found that persons with at least one APOE ϵ 4 allele had significantly lower testosterone levels than those without an ϵ 4 allele. However, from their results it remained unclear how the ApoE protein may interact with testosterone. In the current study, total testosterone levels were not found to differ between APOE ϵ 4 and non-APOE ϵ 4 carriers at their baseline levels. However, there were significantly greater increases in total testosterone levels in the APOE ϵ 4

carriers compared to the non-APOE $\epsilon 4$ carriers, at some of the testing periods during the testosterone treatment (Figure 6.3). However, this result was observed in one arm only (treatment A \rightarrow B), and the trend in the B \rightarrow A treatment group appeared to go in the opposite direction. These unexpected results may be a discrepancy due to the very small number of APOE $\epsilon 4$ participants in the two arms, particularly in the treatment B \rightarrow A group which had only 4 ApoE $\epsilon 4$ carriers, and two of these participants did not respond to testosterone treatment (see Appendices Table 2 and Figure 2). In other studies, Panizzon *et al.* 2010 demonstrated an interaction between testosterone and the possession of APOE $\epsilon 4$ alleles with regards to hippocampal volume in middle-aged men with low testosterone levels. APOE $\epsilon 4$ men with low testosterone levels had the smallest hippocampal volumes. The reason for this is not clear. APOE allelic differences may interact with androgen receptor polymorphisms and/or genes associated with testosterone production. Many more studies are required to elucidate such relationships.

Further studies with larger numbers of participants would be needed to get a clearer picture of any differences in total testosterone levels caused by APOE allele differences. We also pooled the participants from both treatment arms of the study to increase the statistical power (number of participants), but found no significant differences between APOE $\epsilon 4$ and non-APOE $\epsilon 4$ carriers for all of the blood parameters, despite this increase in statistical power.

6.5.2 Effects of testosterone administration on lipid profiles in the plasma

The hormonal regulation of lipid metabolism in men is complex and has not been researched in depth. Sex steroids appear to be key regulatory factors, exerting influence on lipid metabolism in both men and women. However, there is little information in the literature concerning the effects of exogenous testosterone on serum lipids and lipoproteins, especially after prolonged administration. The effects of testosterone on different lipoproteins and lipid levels is of particular interest due to the similarity between cardiovascular disease risk factors and factors thought to influence the risk of AD. The testosterone dosage is also of importance in this issue, as achieving

supraphysiological testosterone levels has been reported to have unfavourable effects on the cardiovascular system (Aaronson *et al.*, 2011) – for example as mentioned below, supraphysiological testosterone has been shown to lower HDL levels. In the experiments reported here, the testosterone dose restored levels of testosterone to an average of 27 nM (see Figures 6.2 and 6.3), and as mentioned at the start of this chapter, the mid-normal range for these Indonesian people is 600-1000 ng/dL, which translates to 21-35 nM. There were differences in responses to testosterone treatment depending on APOE allele status, yet it was found that two of the APOE ϵ 4 participants in Treatment B→A did not respond to the treatment, as mentioned above. Characterising further the effects of testosterone on lipid profiles may shed some light into the possible roles of this hormone in the pathogenesis of AD. Many studies now show there are strong links between lipid profiles (cardiovascular risk factors), APOE allele status and androgen response, and these require further study.

Within the blood, ApoE delivers and transports lipids, especially triglycerides and cholesterol, by mechanisms that involve the binding, uptake and catabolism of lipoproteins. Compared to the APOE ϵ 2 and ϵ 3 alleles, ϵ 4 is associated with poorer transport and clearance of serum cholesterol (Mahley, 1988; Davignon *et al.*, 1988 and Eichner *et al.*, 1993). Researchers have shown that APOE ϵ 4 carriers have higher than average serum cholesterol levels, and interestingly, several animal studies have shown elevated serum cholesterol level are associated with rises in brain A β levels (Kuo *et al.*, 1998; Pappolla *et al.*, 2003). One might assume that there is an effect of APOE allele status causing higher serum cholesterol levels, which would then lead to increased brain cholesterol levels. However, it is important to note that brain cholesterol levels are maintained independently - serum cholesterol levels do not influence brain cholesterol levels directly because cholesterol does not cross the blood brain barrier (Hooijmans *et al.*, 2007). Therefore any increases in brain A β levels may not be due to changes in brain cholesterol *per se*, but rather to effects of increased circulating cholesterol or other influences of ApoE ϵ 4.

In these experiments, no differences were found in total cholesterol, triglyceride, LDL, and HDL results between APOE ϵ 4 carriers and non-APOE ϵ 4 carriers (Figures 6.17, 6.19, 6.21 & 6.23). All of the cholesterol, triglyceride, LDL, HDL, cholesterol to HDL,

and LDL to HDL levels observed in both arms of the study were within normal desirable ranges for Indonesian people in Siloam Hospitals, based on the American Heart Association and National Cholesterol Education Program with total cholesterol ranges being 4.6 - 5.2 mmol/L, triglyceride 0.5 - 1.7 mmol/L, LDL < 3.4 mmol/L and HDL > 1.04 mmol/L. When it comes to measurement of LDL and triglyceride values, a lower level is better because these lipids drive the development and progression of atherosclerosis. In contrast, when it comes to HDL, with few exceptions, a higher level is better because HDL is beneficial and protects patients from the development and progression of atherosclerotic disease (Toth, 2005).

There have been many studies concerning the effects of testosterone on lipid metabolism with conflicting and inconsistent results. Similar to the current study, several studies have found no significant effects on LDL levels (Figure 6.20 & 6.21) but testosterone has been found to result in a mild decrease in HDL levels (Thompson *et al.*, 1989; Freidl *et al.*, 1990; Bhasin *et al.*, 1997). However, several studies have consistently demonstrated an inverse correlation between testosterone and LDL (Saad *et al.*, 2007; Saad *et al.*, 2008; Howell *et al.*, 2001; Ly *et al.*, 2001). The findings in the current study although only showing a trend towards decreasing LDL levels following testosterone treatment are in agreement with these latter studies.

From the results in Figure 6.22 it can be seen that HDL levels were significantly decreased following testosterone treatment, and that these lower levels were maintained until the end of the study. If the participants were classified based on their total cholesterol baseline threshold levels, only those who started off with higher than normal or desirable levels of total cholesterol (moderate) showed significant changes in HDL levels following testosterone treatment, whilst for those participants who initially had low and desirable total cholesterol levels at baseline, it may be that the treatment may not be able to lower levels further, because the body's metabolism will compensate and produce cholesterol to maintain cholesterol levels above a certain minimum level, so these people are not likely to show any change (Appendices).

There have been many studies of effects of testosterone on various aspects of lipid profiles and metabolism, with inconsistent results. In line with the current study's

results, a study by Arver *et al.* (1997) found that transdermal testosterone replacement in testosterone deficient men resulted in a decrease in HDL cholesterol and an increase in the total cholesterol/HDL ratios. Tyagi *et al.*, 1999 also showed that testosterone administration in supraphysiological doses reduced plasma HDL-cholesterol levels, and a study by Handa *et al.*, 1997 showed an inverse relationship between testosterone levels and HDL levels. In other studies, testosterone treatment was found to increase cholesterol, LDL and triglyceride levels (Traish *et al.*, 2009). Furthermore, Malkin *et al.*, 2004 demonstrated that testosterone treatment in hypogonadal men resulted in significant increases in IL-10, a reduction in IL-1 and TNF- α as well as a reduction in total cholesterol and LDL levels, with no significant effect on HDL levels. On the other hand, some studies have shown beneficial effects of testosterone on serum lipids. For example, many epidemiological studies have shown a positive correlation between serum testosterone levels and HDL in healthy men (Khaw, *et al.*, 1991; Freedman *et al.*, 1991). Tenover J.S, (1999) found that testosterone replacement therapy led to an 11% decrease in LDL. In yet other studies, Morley *et al.*, 1993 reported a 9% decrease in total cholesterol levels and no changes in HDL, and a study by Wang *et al.*, 2000 indicated no adverse effects on lipid profile following the administration of testosterone gel.

The clinical significance of a small decrease in HDL levels is still unknown. Serum high-density lipoproteins (HDL) are lower in men than in premenopausal women (Heiss *et al.*, 1980). The enzyme which is primarily responsible for clearance of HDL, hepatic endothelial triglyceride lipase, has been found to have greater activity in men and has been shown to be stimulated by androgens and suppressed by estrogens (Glueck, *et al.*, 1976). Recently, it has been found that a decrease in CAG repeat polymorphisms of the androgen receptor lowers HDL but concurrently lowers the risk of cardiovascular disease (Hersberger *et al.*, 2005). The reason for the discrepancy above may be that the effects of exogenous testosterone on lipids differ from those of endogenous testosterone, doses and formulations of testosterone used, patient age or testosterone deficiency state or that effects may depend on the route of administration (Thompson *et al.*, 1989). Nevertheless, the effects of testosterone on lipid profiles as well as cardiovascular disease remain uncertain.

Cross-sectional studies indicate an inverse relationship between coronary heart disease (CHD) incidence and/or severity and endogenous testosterone levels, irrespective of age (Wu *et al.*, 2003), and testosterone treatment increases coronary blood flow and improved signs on myocardial ischemia in men with CHD (Webb *et al.*, 1999; English *et al.*, 2000). Therefore, total cholesterol/HDL ratios as well as the LDL/HDL ratios were monitored to predict the chances of developing cardiovascular disease. Managing the total cholesterol/HDL ratio below 5:1 is the goal and the ideal one is 3.5 : 1. Results presented in this chapter show no significant changes in total cholesterol/HDL ratios, and the ratios were still below 5 (Fig. 6.24). Also, no significant changes in LDL/HDL ratios (Fig. 6.26) following testosterone treatment were observed which indicates there is no increased risk of cardiovascular disease.

Testosterone has been found to affect estrogen levels that are required to induce apolipoprotein CII and apolipoprotein AI by a mechanism that involves high-affinity androgen receptors (Tam, 1986). In this same study, testosterone was found not to affect the rates of apolipoprotein secretion. At this stage, it appears that few studies have investigated whether testosterone or DHT can influence apolipoprotein production. Other studies have shown that testosterone is lipophilic, suggesting testosterone would be able to intercalate into bilayer cell membranes, potentially altering the fluidity and function of membranes (Whiting, 2000). Considering the low circulating levels of testosterone, this would depend on where and how much the hormone could be concentrated, and factors that would influence where the testosterone would concentrate.

When investigating lipids specifically, animal and human studies have suggested that androgen deficiency is associated with increased triglyceride, total cholesterol, and LDL levels. As mentioned above, similar investigations of HDL levels have been inconclusive, with some people reporting a decrease in HDL levels (Thompson *et al.*, 1989; Bagatell *et al.*, 1984) as found in this study, some showing no change (Zgliczynski *et al.*, 1996; Uyanik *et al.*, 1997), and most of the studies showing increased HDL associated with normalizing testosterone (Saad *et al.*, 2007; Zitzmann and Nieschlag, 2007) . A significant decrease in HDL is a change that would be considered to increase cardiovascular risk. Interestingly, more recent studies of

androgen therapy have shown an increase in HDL levels, which may be associated with improved reverse cholesterol transport (Traish *et al.*, 2009a).

6.5.3 Effects of testosterone on Abeta (A β) levels in plasma

Abeta (A β) peptides, the main constituent of amyloid plaques found in brain in AD, are produced in most tissues and can be detected in the cerebrospinal fluid (CSF) and bloodstream. CSF levels of A β peptides, particularly the more amyloidogenic A β 42 are being considered for inclusion in a panel of biomarkers for AD. Plasma levels of A β peptides are currently thought to be much less reliable, yet as plasma is much more accessible than CSF (which requires lumbar puncture), characterising changes in plasma A β levels is still worthwhile. It may be that A β levels in the bloodstream are influenced by testosterone and our earlier work looking at endogenous testosterone levels support this notion (Bowen *et al.*, 2005).

A β in the blood originates from tissues, or blood components such as platelets (Chen *et al.*, 1995). A β is also produced in the brain (CNS), and can be transported across the blood-brain barrier into the periphery (Zlokovic, 2004) and be detected in plasma. Once in the bloodstream, A β is rapidly taken up by the liver and degraded.

Several studies have demonstrated the diagnostic utility of plasma levels of A β 40 and A β 42. However, the results of these studies are not consistent. (Prvulovic & Hampel, 2011). Our results (Figs. 6.28, 6.29, 6.30 & 6.31) showed no significant changes in plasma A β 40 or A β 42 levels following testosterone treatment, although there was a strong trend towards decreasing A β 42 levels. This results maybe because most of the A β in the plasma is bound to albumin and lipoproteins, with very little free A β (Biere *et al.*, 1996) and thus measured A β levels may be affected by confounding factors (Prvulovic & Hampel, 2011). Interestingly, participants in both treatment arms with APOE ϵ 4 alleles also showed a trend towards reduction of A β 40 following testosterone treatment as opposed to non-APOE ϵ 4 participants (Fig 6.29) which indicates the involvement of APOE in A β clearance.

Plasma A β levels will have been influenced by many factors: age, cerebrovascular disease, liver catabolism, and advanced AD-related pathology are all factors known to affect plasma A β metabolism (Lopez *et al.*, 2008). In addition, lipoproteins in plasma might influence immunological detection of plasma A β (Kawarabayashi and Shoji, 2008). From an assay point of view, this problem is exacerbated by the levels of plasma A β peptide which are about 10-fold lower than in the CSF and by the diversity of ELISA antibodies used (Prvulovic & Hampel, 2011).

Studies by Almeida *et al.* 2004 demonstrated that low levels of testosterone are associated with increased plasma A β levels in men. In relation to Alzheimer's disease, recent follow-up studies have shown that declining plasma A β ₄₂, but not A β ₄₀ levels may be related to AD progression (Mayeux *et al.*, 2003). Before A β deposition in the brain begins, it is hypothesized that high plasma A β levels will be observed, and with the initiation of A β deposition in the brain, it is postulated that A β in the plasma then declines (Locascio *et al.*, 2008).

6.5.4 Effects of testosterone on insulin levels in plasma

The components of the condition known as metabolic syndrome include insulin resistance, glucose abnormality, high blood pressure, obesity or increased waist circumference, and hyperlipidemia. All of the components above are also known risk factors for cardiovascular disease. It has been found that there is an increased risk of testosterone deficiency in men with metabolic syndrome or its individual components, especially insulin resistance, considered by some to be at the core of metabolic syndrome (Guay, 2009). It should not be surprising that testosterone may influence the metabolic syndrome or cardiovascular risk, since testosterone influences lipid metabolism, and can affect a wide range of tissues. For example, vascular cells contain steroid hormone receptors and testosterone can exert effects on vascular walls either directly or indirectly via aromatization (Shabsigh *et al.*, 2005). In addition, androgen deprivation therapy, similar to testosterone deficiency, has been found to increase cardiovascular risk factors and to have adverse effects on cardiovascular function (Traish *et al.*, 2009; Shabsigh *et al.*, 2005).

Based on these findings, there is a strong need to assess the value of testosterone therapy regarding benefits to cardiovascular health. In an early study, testosterone therapy was not associated with the risk of cardiovascular disease in elderly men (Hajjar *et al.*, 1997). Most recent studies have found that many indicators of cardiovascular health, such as high blood pressure, glycemic control, insulin resistance and high LDL levels are altered favourably on treatment with testosterone (for example, Jones *et al.*, 2011).

However some studies have reported transient improvements or no change in one or more variables. For example, data on the effect of testosterone replacement therapy on insulin sensitivity have yielded conflicting results. Some studies have reported favorable effects in men with obesity (Marin *et al.*, 1992) or type 2 diabetes (Kapoor *et al.*, 2006) and in healthy older men (Emmelot-Vonk *et al.*, 2008). Contradictively, other studies have shown no changes in insulin sensitivity on healthy young and older men. Other cross-sectional studies have shown a negative correlation between total testosterone and insulin levels in the plasma (Seidell *et al.*, 1990; Pasquali *et al.*, 1991; Simon *et al.*, 1992). The current study showed some effects of testosterone on insulin sensitivity, although no strong correlation was observed (Figs. 6.32 & 6.33). Perhaps if this study was repeated with a much larger cohort, statistically different results would be obtained.

More recently, epidemiological studies have suggested that low levels of total testosterone and SHBG can be considered to be predictive of metabolic syndrome and to increase the risk of diabetes Type II (Saad, 2009). Low testosterone levels appear to precede elevated fasting insulin, glucose, and hemoglobin A1c (HbA1C) values and have been associated with increased deposition of visceral fat, which serves as an endocrine organ, producing inflammatory cytokines and thus promoting endothelial dysfunction and vascular disease (Traish *et al.*, 2009c). In fact recent comprehensive reviews have found that testosterone treatment may be able to slow the progression from metabolic syndrome to diabetes Type II or cardiovascular disease, via beneficial effects on insulin regulation, lipid profile and blood pressure (Saad *et al.*, 2011; Makhsida *et al.*, 2005). The positive effects that androgen replacement therapy may have in combating obesity in testosterone deficient men remains largely unknown to medical professionals, partly because of the (incorrectly) perceived increased risk of prostate cancer. The reports of myocardial infarction, hypertension, arrhythmia, cardiac

failure, pulmonary embolism and stroke in men following androgen abuse have also not helped to promote the restoring of normal levels of testosterone (Weidemann and Hanke, 2002).

Recent studies suggest that long-term treatment with low doses are safe (Miner *et al.*, 2008). Due to studies of individuals keen to achieve supraphysiological testosterone levels, it has nevertheless been suggested that there may be an ideal testosterone “physiological window”: within this window testosterone has favourable effects, but above or below the safe window these androgens can have a negative effect on the body (Blouin *et al.*, 2008; Basaria *et al.*, 2010). These reported negative effects of supraphysiological levels of testosterone are likely deterring physicians from considering testosterone therapy for the treatment of androgen deficiency.

Future studies are required to determine how hormone changes and hormone therapy can help reduce the health problems associated with the increasing number of people suffering from metabolic syndrome, and determine if such treatment can also reduce the risk or development of AD.

6.5.5 Effects of testosterone on Red Blood Cell (RBC) counts and Hemoglobin levels

Androgens have long been known to stimulate red blood stem cell numbers (Shahidi, 1973). Several androgen replacement therapy treatments have reported significant increases in hemoglobin and hematocrit levels in a dose-dependent manner (Hajjar, *et al.*, 1997; Sih *et al.*, 1997; Bachman *et al.*, 2010; Covellio *et al.*, 2008), with greater increases in red blood cells in older men than in young men (Covellio *et al.*, 2008).

The results in the current study are in agreement with these previous studies, as they show that the administration of testosterone enhances red blood cell levels in the plasma (Figure 6.36 and Figure 6.37). It has been found that testosterone treated-men are nearly four times as likely to have hematocrits of >50% as compared to placebo-treated men (Calof *et al.*, 2005). Therefore, it is important to note that testosterone treatment should not be administered to men with baseline red blood cells or hematocrit of 50% or greater without appropriate evaluation and treatment of erythrocytosis (Bhasin *et al.*, 240

2010) since testosterone treatment can worsen pre-existing erythrocytosis. Although it had been postulated that testosterone stimulates erythropoiesis by modulating erythropoietin and stem cell proliferation, recent data suggests that testosterone increases red cell mass by inhibiting hepcidin (Bachman *et al.*, 2010).

Testosterone deficiency has also been associated with lower hemoglobin levels, as shown by the lower normal range for haemoglobin in women. In line with studies by McCullagh *et al.*, 1942 and Hamilton *et al.*, 1964 which showed that testosterone replacement restores the red blood cell counts, this study has also found a significant increase in hemoglobin levels following testosterone treatment (Figs 6.38 & 6.39), similar to that seen in the results of the red blood cell levels. The normal range of hemoglobin in men is 14-18 ng/dL (Billet, 1990), and although significant increases in hemoglobin were observed following testosterone treatment, the levels seen in our results were still in the safe range in all of the treatment groups in both arms.

6.5.6 Effects of testosterone on Prostate Specific Antigen (PSA) levels in plasma

In the past there have been major concerns regarding testosterone therapy promoting the growth of metastatic prostate cancer. However, it has now been established that there is no evidence of increased risk of prostate cancer (Jackson, 2012; Feneley and Carruthers 2012 pub on-line JSM). The general view now is that testosterone does not promote prostate cancer but can exacerbate existing cancer (Fowler *et al.*, 1981; Bhasin *et al.*, 2003) and should not be administered in men with already high PSA levels (Bhasin *et al.*, 2010). In fact in one study an increased risk of prostate cancer has been associated with low plasma level of testosterone (Raynaud, 2006). Therefore, testosterone therapy should be accompanied by a standardized monitoring plan which includes periodic ascertainment of prostate specific antigen (PSA) levels and digital prostate examination. Most studies have shown no significant increases in PSA or prostate volume following testosterone administration to testosterone deficient men (Behre, H.M., 1994).

The results in this Chapter (Figs 6.34 & 6.35) show a significant increase in PSA levels following testosterone treatment, although levels still remained within the safe and normal range, which is 0-4 µg/L (American Cancer Society). According to the

American Cancer Society, a PSA level between 4 and 10 µg/L indicates a 25% risk of having prostate cancer. A PSA higher than 10, increases the risk of having prostate cancer to 50% and above. Since PSA levels tend to increase with age, the use of age-specific PSA reference ranges has been suggested to improve the accuracy of PSA tests (National Cancer Institute). In accordance with our study, Khera *et al.*, 2011 have shown a small increase in PSA levels following 12 months of testosterone treatment. A number of open-label trials have also reported that testosterone treatment results in very low rates of developing prostate cancer (Agarwal *et al.*, 2005; Kaufman *et al.*, 2004). Although PSA levels were higher in the testosterone treated group compared to placebo, these results was not found to be significant (Calof *et al.*, 2005). Interestingly, subjects with APOE ε4 alleles had higher levels of PSA than non-APOE ε4 subjects (Fig. 6.35). All of the participants in both arms had normal PSA levels at baseline (Appendices).

6.5.7 Effects of testosterone on Body Mass Index (BMI) and body fat percentage

According to the World Health Organization (WHO), the normal reference range for BMI is 16-19 kg/m² for the Indonesian population. A BMI of over 22 kg/m² is considered to be overweight and a person with a score over 27 kg/m² is considered obese. Therefore, according to their BMI levels, most participants in both arms of this study were considered to be overweight (see Appendices Table 7 & 8). Although no significant changes in BMI levels were observed in any of the treatment groups, most of the participants showed an increase in BMI following testosterone treatment at individual levels in both arms (see Appendices). However, it is important to note that BMI includes both the body fat mass as well as body muscle and there was no differentiation between the two.

The WHO has concluded that the Asian population generally has a higher percentage of body fat than Caucasian people of the same age, sex, and BMI. However, there is no reference range of body fat percentage in Asian populations to date. Gallagher *et al.* 2000 established that people aged 41-60 with an age-adjusted body fat percentage of over 27% should be considered to be obese. Based on this reference, only 5 participants in this study should be considered to be obese at baseline (see Appendices Table 9 & 10). Although there were no significant differences in body fat percentage observed

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following testosterone treatment, most of the participants showed a trend towards increasing body fat at individual levels in both treatment arms. Taken together, an increased BMI and body fat percentage in these men following testosterone treatment suggests most of the participants produced more fat cells compared to muscle mass. This result may be due to the fact that most of the participants were already overweight at baseline.

Conclusions

In conclusion from this Chapter, testosterone replacement therapy for 6 months in testosterone deficient men caused a significant increase in testosterone and DHT levels (but not estradiol) as expected, a significant decrease in LH, a decrease in HDL, a trend towards increasing plasma insulin, and a significant increase in the number of red blood cells and hemoglobin. No changes in estradiol, total cholesterol, triglycerides, or LDL levels were found. The slight increase in PSA levels is unlikely to be sufficient to influence risk of prostate changes. A β 40 and A β 42 levels in the plasma were not significantly affected by testosterone treatment, although, there seemed to be an encouraging trend towards decreasing levels of A β 42 following testosterone treatment. With the increasing number of people suffering from metabolic syndrome, future studies are required to determine how hormone changes and hormone therapy can help reduce the health problems associated with metabolic syndrome, and determine if such treatment can also reduce the risk or development of AD.

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Chapter 7

Effect of testosterone supplementation on cognitive performance, depression and quality of life in men with subjective memory complaints (SMC)

7.1 Introduction

Aging is associated with a general decline in many cognitive functions, with the rate of decline varying between individuals. Serum testosterone levels also decline with aging in men after 30 years of age (Harman *et al.*, 2001), decreasing at an annual average rate of 0.2-1% for total testosterone and 2-3% for free testosterone (Feldman *et al.*, 2002). It has been shown in many laboratory-based studies that sex steroid hormones such as estrogens and testosterone, have neurotrophic and neuroprotective properties, and many clinical reports suggest these hormones could provide neuroprotection during aging (Lee *et al.*, 2001; McEwen *et al.*, 1997; Torrand-Allerand *et al.*, 1992). Similarly, age-related declines in testosterone levels have recently been associated with declines in cognitive function, such that low testosterone is now believed to be a risk factor for cognitive decline. Elderly men generally have higher sex hormone levels than elderly women (Hogervorst *et al.*, 2001; Hogervorst *et al.*, 2004). Therefore, theoretically, aging men should preserve certain aspects of cognitive function to a better extent than aging women. Studies of elderly subjects which have investigated the relationship between cognitive function and endogenous as well as supplemented (hormone replacement therapy) steroid hormone levels have provided some support for this theory, however these relationships also seem to be modified by other gender-related factors (Hogervorst *et al.*, 2007)

There has been extensive research into the association between the age-associated decline in testosterone levels in men and their performance in various cognitive measures. Serum levels of testosterone have been found to correlate with cognitive performance in a number of neuropsychological tests in elderly men. Cross-sectional studies have shown positive associations, such that higher levels of bio-available testosterone, but not of bio-available estradiol, are associated with better cognitive function in older men (Yaffe *et al.*, 2002). Moreover, there were curvilinear associations between total testosterone and verbal memory (Muller *et al.*, 2005) as well as between free testosterone and attention or working memory (Barret-Connor *et al.*, 1999; Matousek *et al.*, 2010), and global cognition (Yeap *et al.*, 2008). These curvilinear associations suggests an optimal testosterone level for particular cognitive tasks. Meanwhile, these curvilinear associations may contribute to negative associations (Hogervorst *et al.*, 2004; Martin *et al.*, 2007) as well as to non-significant associations

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(Wolf *et al.*, 2002; Carlson *et al.*, 2000). These associations further suggest that there is an optimal level for testosterone, above or below which there is no further improvement in cognition and more likely there will be negative effects. However, there are many longitudinal studies which have reported no significant associations between testosterone and cognition (Moffat *et al.*, 2002 ; Hogervorst *et al.*, 2010). All these studies have produced a variety of results, and this is most likely due to significant differences in age ranges, types of cognitive tests used, cohort and sampling methodologies, inclusion and exclusion criteria, different assays for measuring testosterone, and the absence of follow-up testosterone measurements.

Testosterone has demonstrated neuroprotective effects in laboratory-based studies. Such results from cell culture and animal studies indicate that testosterone could also provide neuroprotection in the brain, especially in those regions that are susceptible to AD pathogenesis. Such regions include the hippocampus and cortical regions which have a high density of androgen receptors (Simerly *et al.*, 1990). In the brain, testosterone can mediate its neuroprotective effects directly through binding to androgen receptors, or following conversion to estradiol (Pike *et al.*, 2009). There is growing evidence to support these neuroprotective effects of testosterone. However, the effects of testosterone on the brain in terms of cognitive function are not fully understood and the available data are not conclusive.

Beneficial effects of testosterone supplementation have been reported previously (Alexander, *et al.*, 1998; Janowsky, *et al.*, 1994; Klaiber, *et al.*, 1971). However, testosterone supplementation in testosterone deficient men have not always demonstrated any significant effects on cognition (Ly *et al.*, 2001; Cherrier *et al.*, 2003; Kenny *et al.*, 2002; Kenny *et al.*, 2004; Vaughan *et al.*, 2007; Sih *et al.*, 1997).

On the other hand, testosterone depletion does tend to increase depression. A study by Almeida *et al.* (2003) showed that chemical castration resulting in testosterone depletion caused an increase in depression and anxiety. This suggests that age-related depression may be due to the age-related declines in testosterone levels. There are studies which suggest that depression and the development of AD may be linked: for example, two studies (Dal Forno *et al.*, 2005; Fuhrer *et al.*, 2003) have demonstrated that a relationship between depression and a risk for AD mainly exists in men.

Therefore, current depression levels and any history of depression need to be considered carefully when assessing elderly men and their risk of AD.

The role of estradiol, luteinizing hormone (LH), and genetic factors when considering AD risk in men is also not fully understood. During normal aging, testosterone levels gradually decline, but estradiol levels do not change significantly (Rosario *et al.*, 2009 ; Twist *et al.*, 2000). Several observational studies have shown negative associations between high estradiol levels and cognition. There is a possible role for gonadotropin levels that may further modify the role of testosterone in relation to cognition and brain function. The LH levels may have a negative association with cognition (Hyde *et al.*, 2010). The potential role of genetic factors is also of interest. In particular, the optimal level of testosterone may be influenced by APOE genotype. In male non-APOE $\epsilon 4$ carriers, testosterone levels have been found to correlate with performance on cognitive tests that investigate working memory, attention and executive functioning, whereas in men carrying APOE $\epsilon 4$ alleles, higher testosterone concentrations have been associated with a lower performance in such cognitive tests (Burkhardt *et al.*, 2006).

This chapter shows results of the assessment of the effect of transdermal testosterone supplementation on cognitive performance, verbal memory, depression, as well as quality of life in testosterone-deficient elderly men. This study assessed the efficacy of testosterone in improving cognitive function during a six month treatment trial while statistically controlling for the effects of age and education.

7.2 Aims

The aims of this study were :

- To investigate the effects of testosterone administration on global cognitive functioning;
- To investigate the effects of testosterone administration on verbal memory;
- To investigate the effects of testosterone administration on depression;
- To investigate the effects of testosterone administration on health-related quality of life which constitutes both physical and mental health;

- To investigate whether there are different responses to testosterone treatment in relation to cognition, depression, and quality of life in the presence of APOE ϵ 4 allele.

7.3 Methods

Participants

Participants were healthy older men with low levels of testosterone, aged 50 to 75 years old (mean age 61 years \pm 8 years) who had some concerns about their memory functioning and were recruited from the community through flyers. The study protocol was approved by the Edith Cowan University (Western Australia), and the Faculty of Medicine, University of Indonesia Independent Ethical Committee, and approved informed consent procedures were followed. Details of the study design are described in section 6.3. Participants underwent a screening assessments at the baseline visit to determine eligibility criteria as described in section 2.1.1.1. The following eligibility criteria were used: 1) Males 50 years of age or older; 2) A Mini Mental State Examination (MMSE) score of 24 or above; 3) A normal range of prostate-specific antigen (PSA); 4) Blood pressure within normal limits, 110/75 – 130/85 5) No history of diabetes mellitus; 6) Normal liver and kidney enzyme function; and 7) No history of major head injury, based on the medical track record. Apart from blood tests to measure plasma testosterone levels, participants also underwent a cognitive screening test (MMSE) at baseline. Participants with MMSE scores below the recommended cut-off score (\leq 24) were excluded from the study.

Neuropsychological test measures

In clinical practice, neuropsychological tests have always been fundamental in the clinical diagnosis of AD and other dementias. The recent development of potential drug treatments and preventative therapies has raised the issue that earlier diagnosis, as well as a more solid framework of neuropsychological assessment of AD are required. The majority of current tests have an acceptable sensitivity and specificity for screening of mild to moderate stage of AD. For example, the MMSE shows a sensitivity of 87% and specificity of 92% with the cut-off score 24 (Grut *et al.*, 1993) and the Rey Auditory Verbal Learning Test (RAVLT) can distinguish between AD patients and those without

dementia, or between AD and other forms of dementia with a diagnostic accuracy of 83-86% (Gilhus *et al.*, 2011).

In these studies, the cognitive test battery consisted of the MMSE and the RAVLT. The MMSE is a brief cognitive measure which has been translated into many languages to assess memory, attention, language, orientation, aphasia and apraxia and it is widely used in clinical trials and research (Dufoil *et al.*, 2000).

The RAVLT evaluates a wide diversity of functions: short-term auditory-verbal memory, rate of learning, learning strategies, retroactive, and proactive interference, presence of confusion in memory processes, retention of information, and differences between learning and retrieval. The test was administered to each participant according to standard procedure (Lezak, 1983). The RAVLT consists of a list of 15 unrelated words that need to be remembered. After presentation of Trial 1 – Trial 5, followed by free recall of the words immediately and consecutively, participants were presented with an interference list of 15 words. After free recall of interference list, participants were asked to recall words from the original list without additional presentation of the words (Trial 6). The last recall trial was followed by a 20-minute interval (Trial 7). Finally, participants were presented with word lists for recognition consisting of 15 words from list A, 15 words from list B, and 20 words as distracters. Participants were to indicate words they recognized. The recognition measures used in this study include: List A recognition score (true positives), the number of words correctly identified from List A; List B recognition score, the number of words correctly identified from List B; distractors correctly identified score (true negatives); and the number of words mistakenly identified from either List A or List B. The application of RAVLT list of words in this study used parallel forms of the RAVLT (matched for equal difficulty) to ensure that retesting could be conducted over time without the memory data being potentially confounded by practice or learning effects. RAVLT Test type A, B, C, and then D were administered starting from the baseline until the end of the study.

Depression levels were measured by the Geriatric Depression Scale (GDS). The GDS has demonstrated an acceptable specificity and sensitivity, and is widely used for depression screening in the elderly (Yesavage *et al.*, 1983 ; Segulin, 2007). The GDS

scores range from 0-30 with a score of ≥ 11 as the cut-off score for depression. A higher score is indicative of a higher depression level.

Health-related quality of life was measured using the Short-Form Health Survey (SF-36). The SF-36 comprises 36 items measuring 8 different dimensions including physical functioning, physical status, mental health, emotional status, social functioning, vitality, pain, and general health perceptions. From the results, two aggregate scores can be generated which indicate physical health and mental health. The scores on each scale range from 0 (the worst health state) to 100 (best health state) (Ware *et al.*, 1992).

The MMSE and SF36 tests were conducted at baseline and repeated at week 24 (end of treatment period), week 28 (washout period), and week 52 (end of cross-over period). The RAVLT and GDS tests were conducted at baseline and were repeated every 2 months following the first treatment period, the washout period, and following the second treatment period (cross-over period). All neuropsychological tests were translated into Indonesian and have been validated in Indonesian hospitals. Physicians, participants, and investigators were blind to the treatment conditions.

APOE genotyping

At the end of cognitive and clinical assessment sessions, whole blood samples were collected and were separated into various components using standard centrifugation techniques. White blood cells were used for APOE genotyping, which was determined using PCR as already described in section 2.1.1.2.3.

Statistical analysis

Statistical analysis was performed using The Statistical Package for Social Sciences (SPSS version 19, SPSS Inc, Chicago, IL). The Kolmogorov-Smirnov and Levene's tests were used to examine normality of distribution of continuous variables for each group. The data from the MMSE, RAVLT, GDS, and SF36 tests were found to be normally distributed. Therefore, one-way repeated measures ANOVA were performed. Carry-over effects were tested from differences at baseline in the first period (week 0) and at the washout period (week 24-28, which was baseline for the second period). Statistical analyses were performed to look at changes in general cognitive function,

memory, depression level and quality of life over time and in various groups. When directly comparing two groups from both arms of the study, as well as comparing two groups based on the presence of APOE-ε4 alleles, two tailed independent t-tests were used. Correlations of cognitive performances were assessed by Pearson product-moment correlation coefficient (r) for parametric data, whilst Spearman (rho) was used for non-parametric data. All analyses were two-tailed and the alpha level was set at $p < .05$

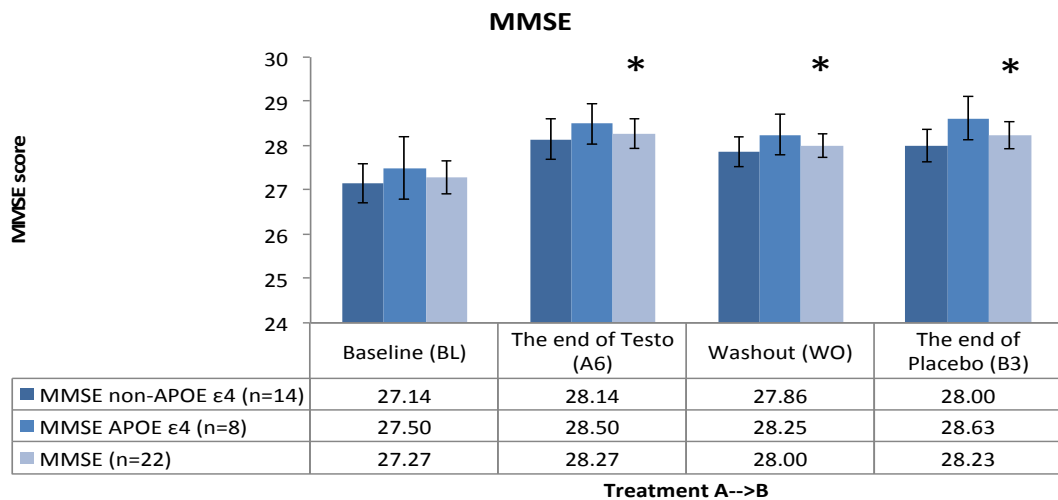
7.4 Results

Table 7.1 Mean (SD) age and education

	Non-APOE ε4	APOE ε4	p value
Treatment A-->B			
Age	59 (5.82)	59.625 (9.58)	0.87
Education	13.79 (3.31)	14.25 (1.91)	0.68
Treatment B-->A			
Age	62.72 (7.31)	63.75 (13)	0.88
Education	13.47 (3.62)	15 (2)	0.25

Based on the statistics above, neither age nor education was significantly different when comparing any of the groups. The p value indicates that there were also no significant differences in age or education between non-APOE ε4 and APOE ε4 carriers. Therefore, age and education would not be expected to influence the following statistical analyses in this study.

(A)



(B)

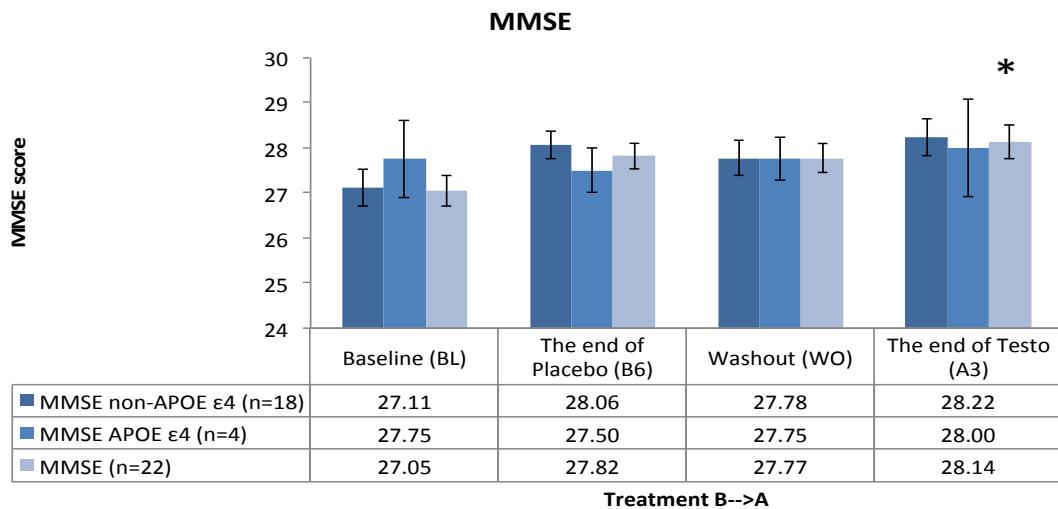
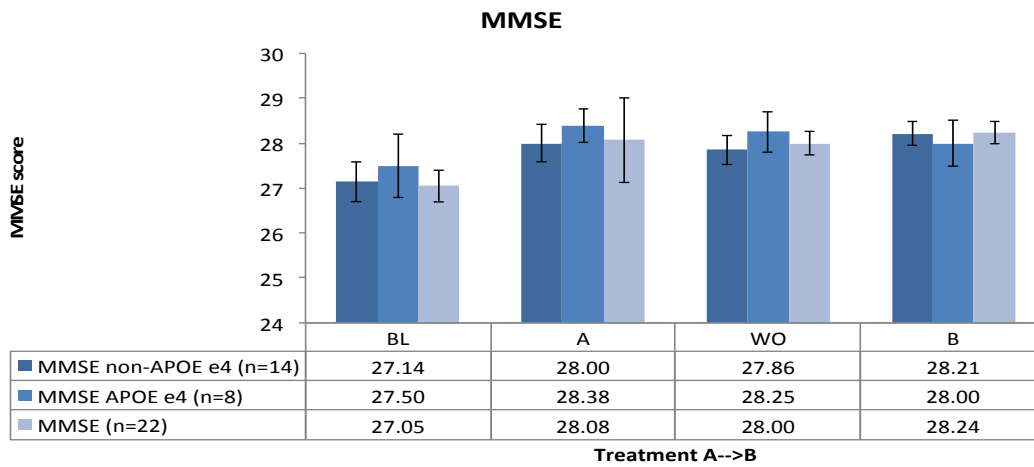


Figure 7.1 (a) : The MMSE score for treatment A→B (A) and treatment B→A (B) at 4 different time points : BL= Baseline (Week 0), A₆ & B₆ = End of First Treatment period (Week 24), WO=Wash-out (Week 28), B₃ & A₃ = End of Second Treatment or Cross-over period (Week 52). Bar represent mean and standard deviation (\pm), with values underneath the bars for the mean of each groups. MMSE score was significantly improved after testosterone treatment in both arms and after washout and placebo treatment in treatment A→B. * $p < 0.05$ in comparison to baseline of each arm of the treatments. No significant differences were observed between non-APOE ϵ 4 and APOE ϵ 4 group.

(A)



(B)

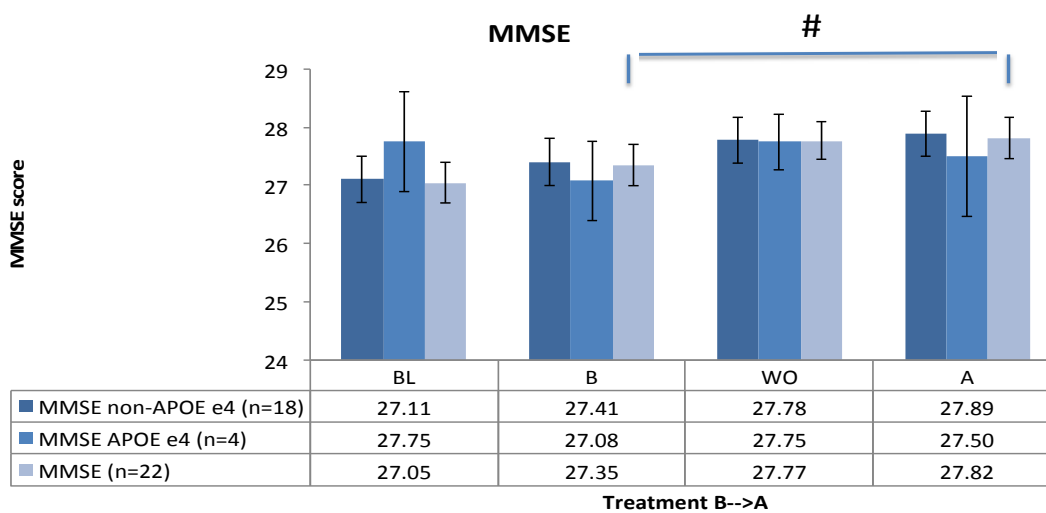


Figure 7.1 (b): The MMSE score for treatment A→B (A) and treatment B→A (B) at 4 different time points : BL= Baseline (Week 0), A_ = Mean of Testosterone treatment, WO=Wash-out (Week 28), B = Mean of Placebo treatment. Bar represent mean and standard deviation (\pm), with values underneath the bars for the mean of each groups. MMSE score was significantly improved following testosterone treatment compared to placebo in treatment B→A only, regardless of APOE (Graph B). # $p < 0.05$ in comparison between groups. No significant differences were observed between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ group.

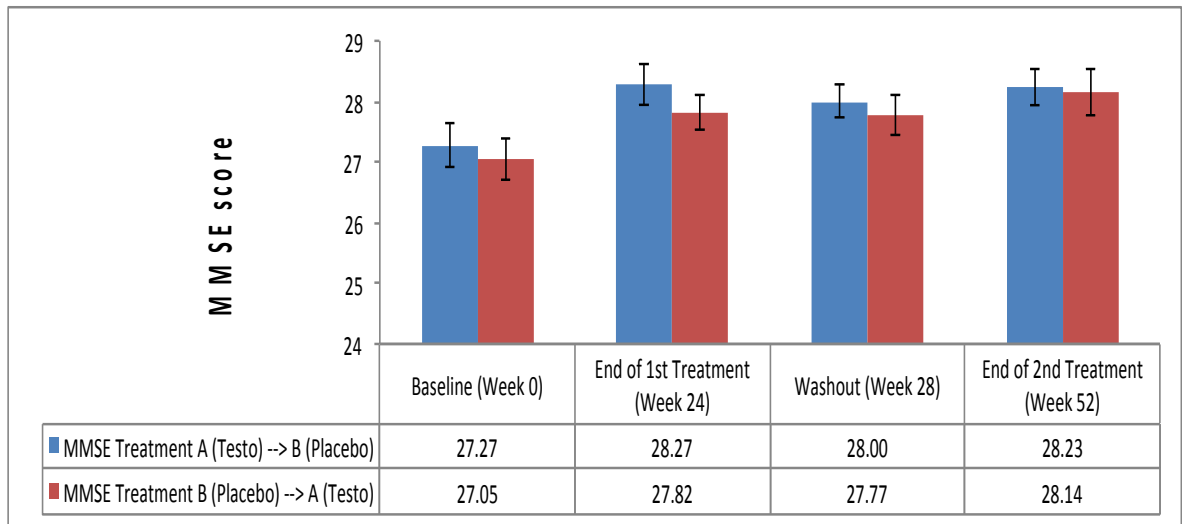
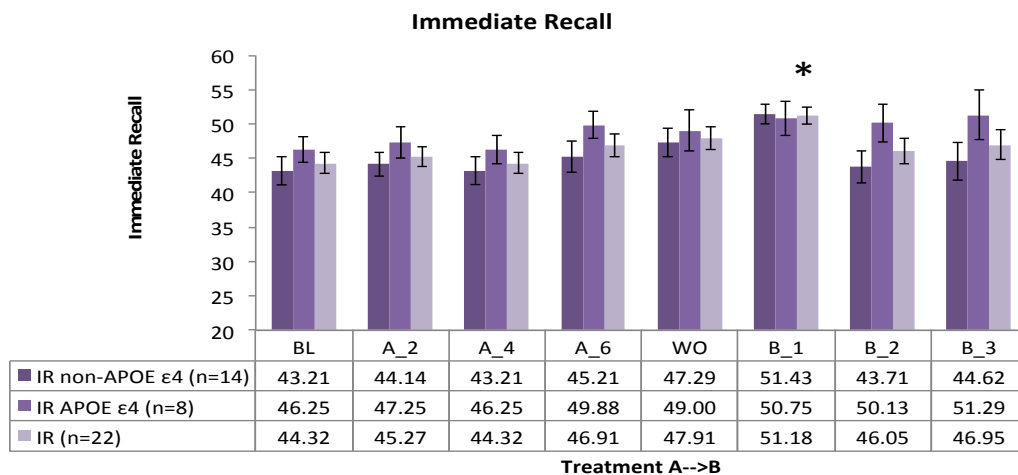


Figure 7.2: MMSE score comparison between treatment arm A→B and treatment B→A. Bars represent the means \pm standard deviation, with actual mean values shown underneath the bars of each group. No significant differences were observed between any of the groups. N=22 for each arm.

(A)



(B)

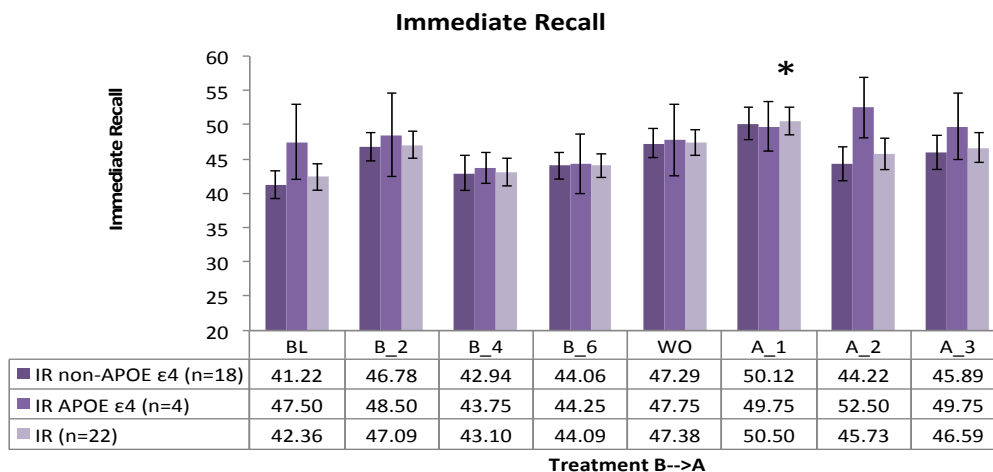
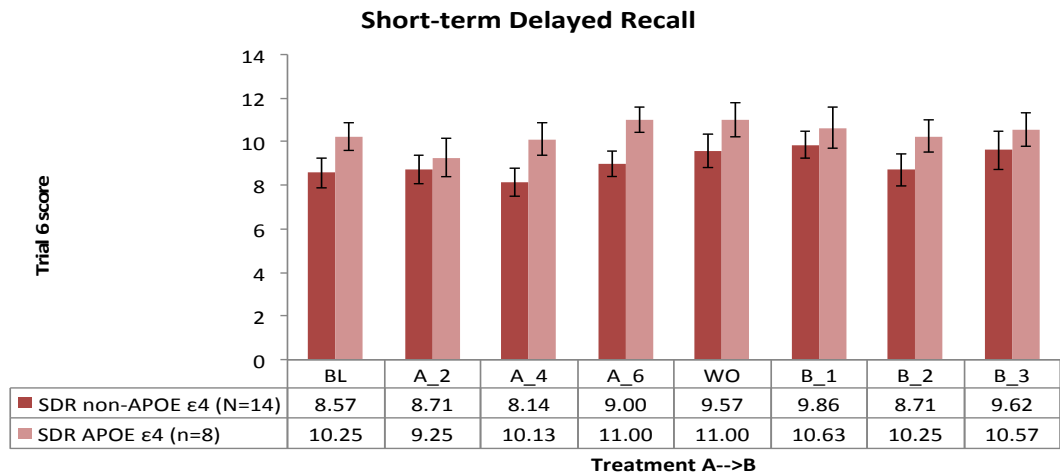


Figure 7.3 : Immediate Recall (Total Learning from Trial 1 to Trial 5) score for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean and standard deviation (\pm), with values underneath the bars for the mean of each group. Immediate recall score (as measured by RAVLT) was significantly increased over time at visit 9 (week 36) in both arms, regardless of APOE (Graph A and B) * $p < 0.05$ in comparison to baseline for each arm of the treatments. No significant differences were observed between non-APOE ε4 and APOE ε4 groups. However, participants with APOE ε4 tend to recall the words better than non-APOE ε4 participants.

(A)



(B)

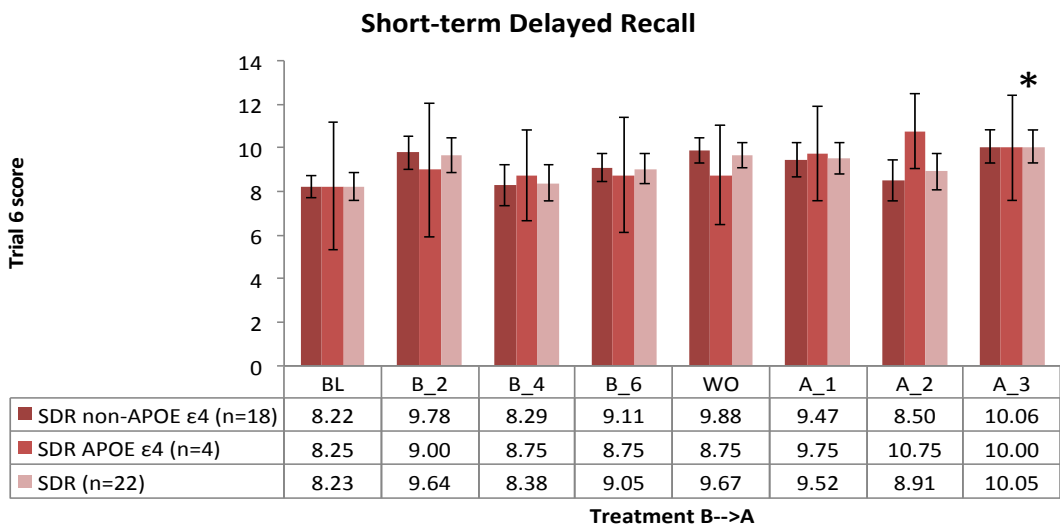
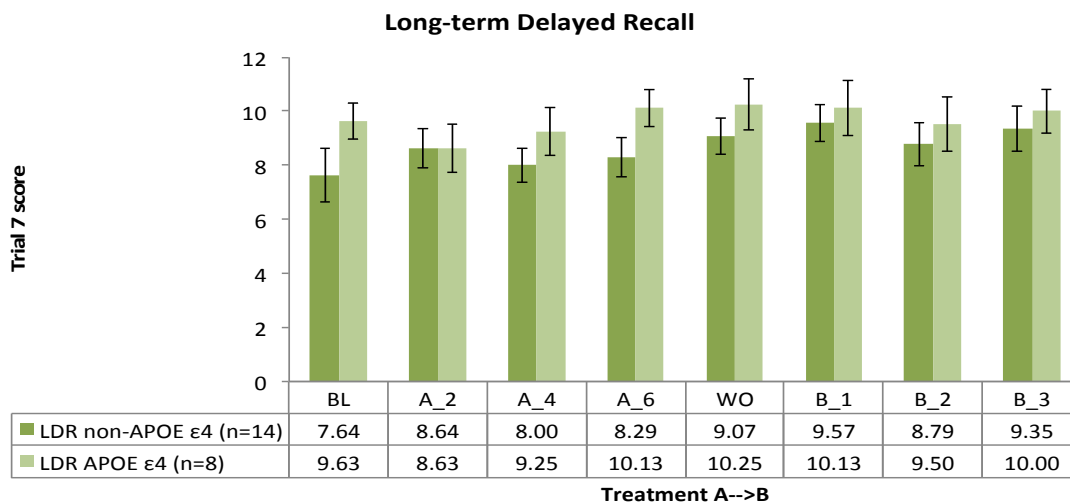


Figure 7.4 : Short-term delayed recall for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. Short-term delayed recall score (as measured by Trial 6 RAVLT) was significantly increased at the end of testosterone treatment compared to baseline (* $p < 0.05$) at treatment B→A only, regardless of APOE (Graph B). No significant differences were observed between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ group.

(A)



(B)

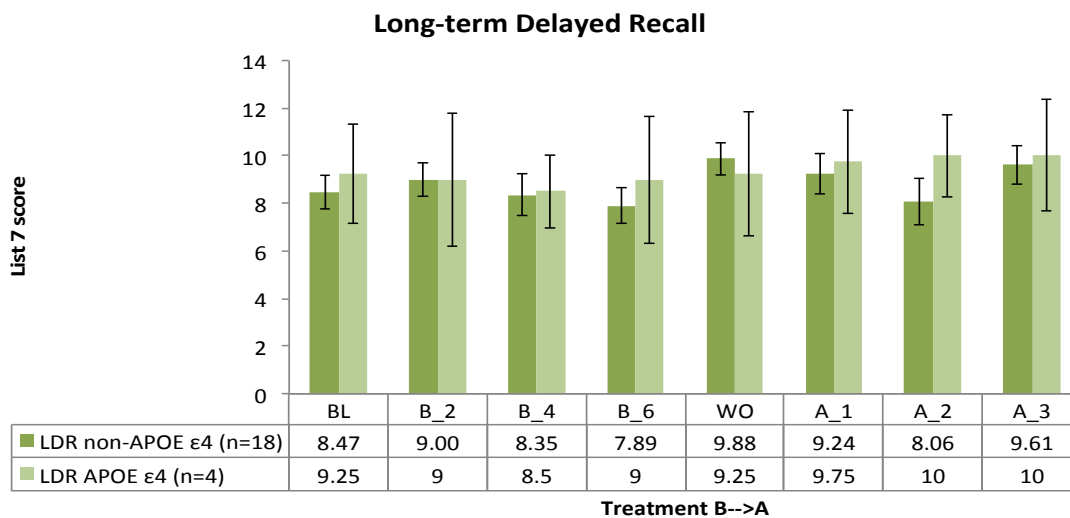
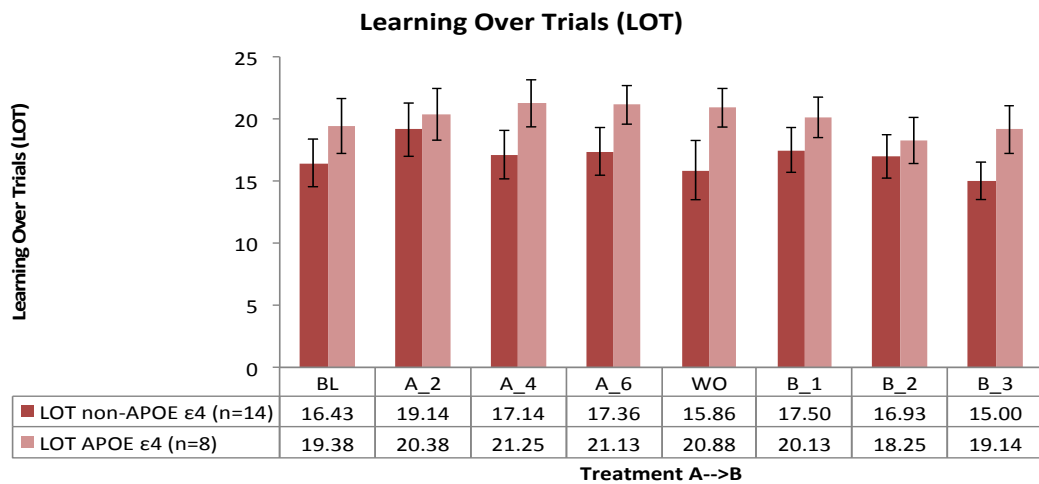


Figure 7.5: Long-term delayed recall for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean \pm standard deviation, with values underneath the bars for mean of each group. There were no significant differences in long-term delayed recall score (as measured by Trial 7 RAVLT) between any of the groups of either arm. No significant differences were observed between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ groups.

(A)



(B)

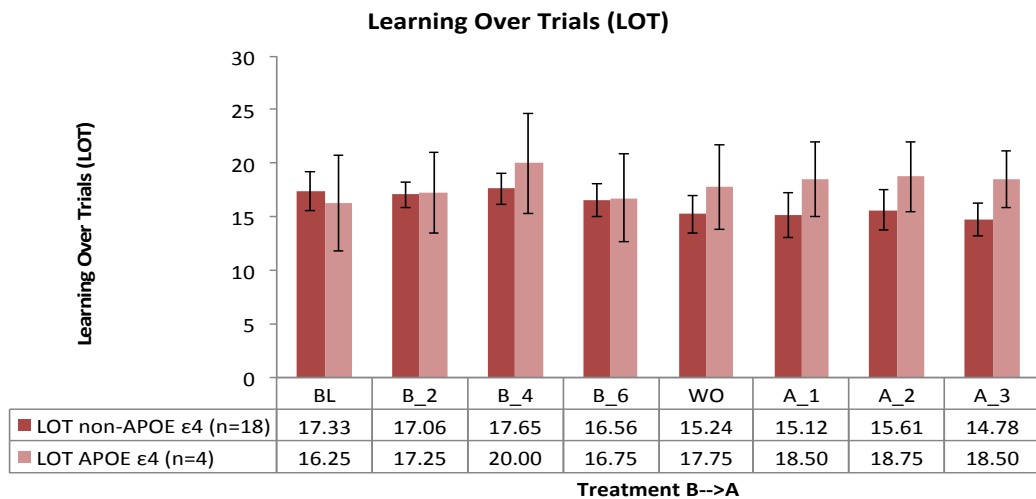
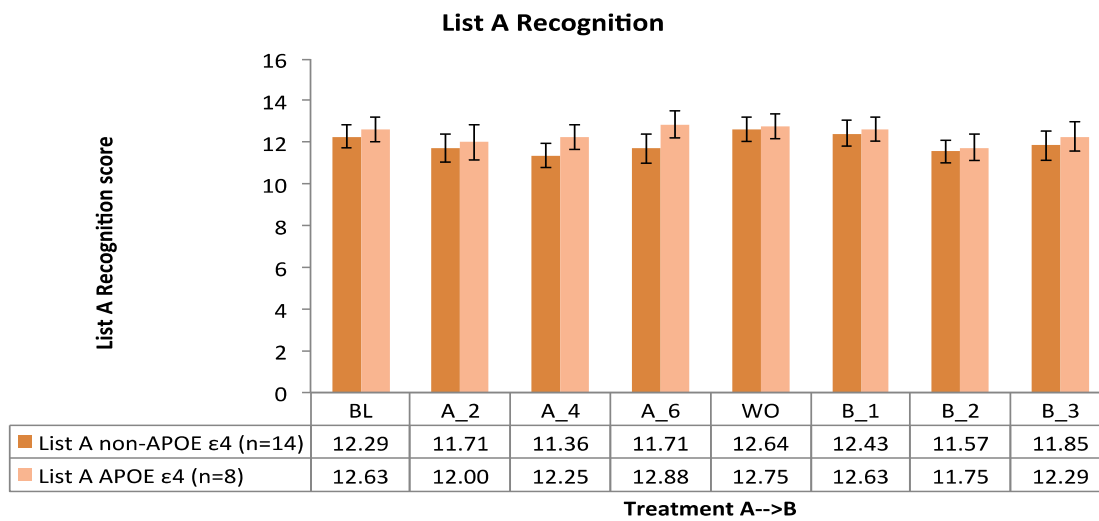


Figure 7.6: Learning Over Trials (LOT) score for treatments A→B (A) and B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (A) & B_2 to B_6 (B) = First Treatment period , WO=Wash-out, B_1 to B_3 (A) & A_1 to A_3 (B) = Second Treatment or Cross-over period. LOT = Total Learning – (5 x Trial 1) (Tierney *et al.*, 1994). Bars represent mean \pm standard deviation, with mean values shown below the bars. LOT score (as measured by RAVLT) was significantly increased following testosterone treatment compared to the end of placebo treatment in treatment A→B (A). # $p < 0.05$ in comparison between groups. No significant differences were observed between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ groups. However, APOE $\epsilon 4$ participants tended to have a higher learning effect than non-APOE $\epsilon 4$ participants.

(A)



(B)

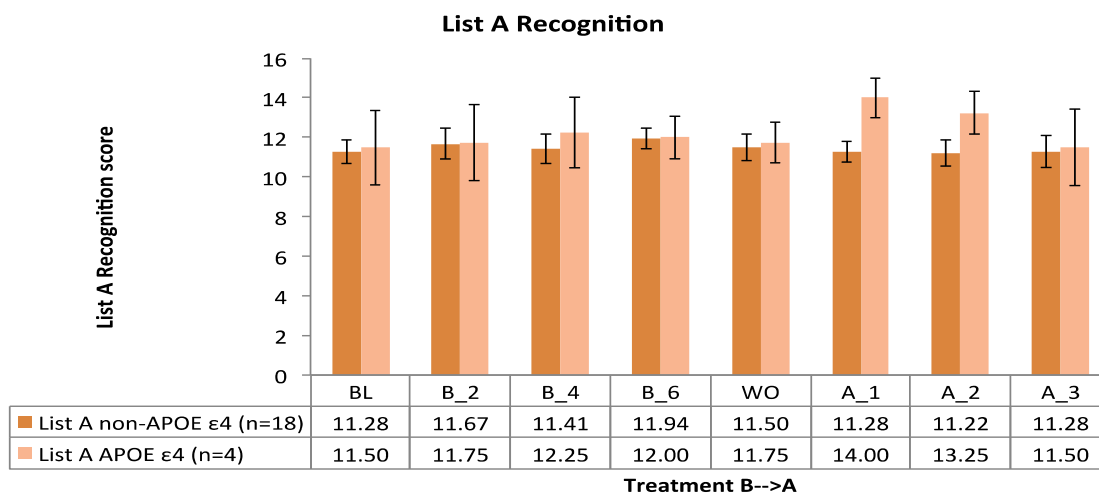
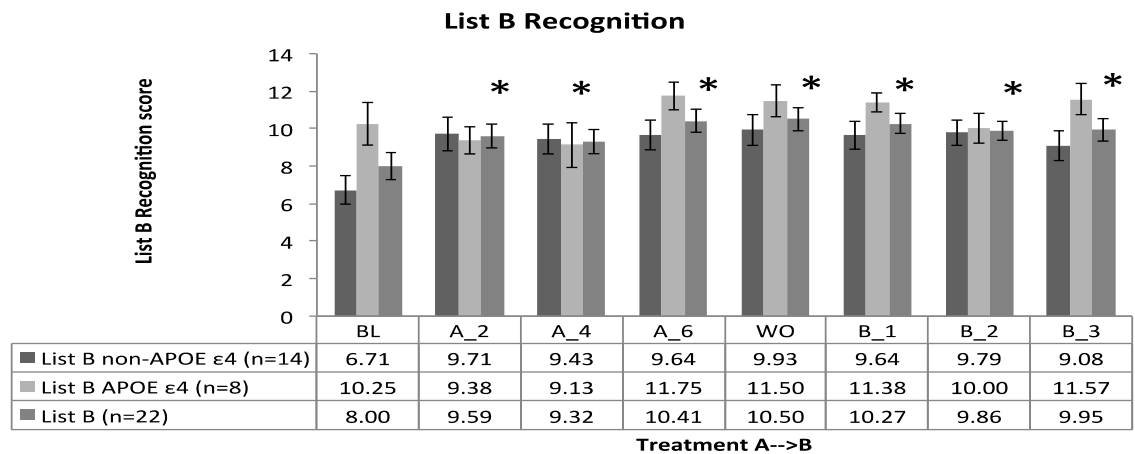


Figure 7.7 List A Recognition score for treatments A→B (A) and B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (A) & B_2 to B_6 (B) = First Treatment period , WO=Wash-out, B_1 to B_3 (A) & A_1 to A_3 (B) = Second (Cross-over) Treatment. Bars represent mean \pm standard deviation, with mean values underneath the bars for each group. There were no significant differences observed in List A recognition (as measured by RAVLT) between any of the groups of either arm (A and B). No significant differences were observed between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ groups. However, APOE $\epsilon 4$ participants tended to recognize words from list A better than non-APOE $\epsilon 4$ participants after testosterone treatment.

(A)



(B)

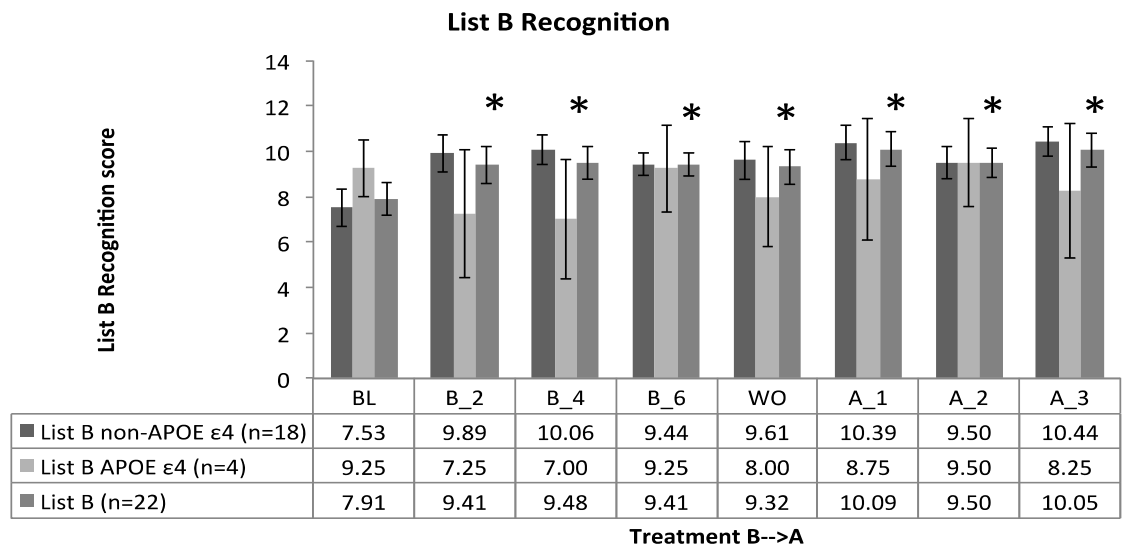
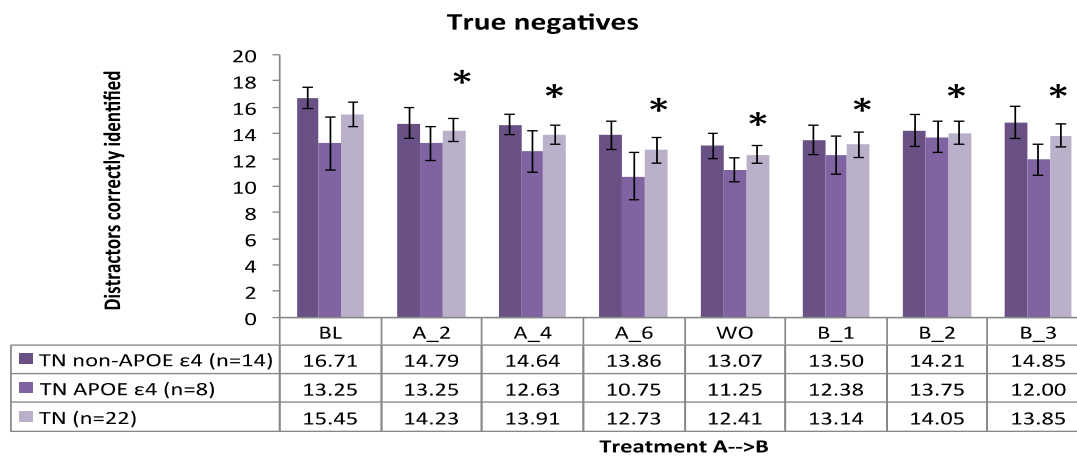


Figure 7.8 : List B Recognition score for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. There were significant differences of List B recognition (as measured by RAVLT) observed in all of the groups of both arms, regardless of APOE (Graph A and B). No significant differences were observed between non-APOE ε4 and APOE ε4 group (Sodergard, 1987).

(A)



(B)

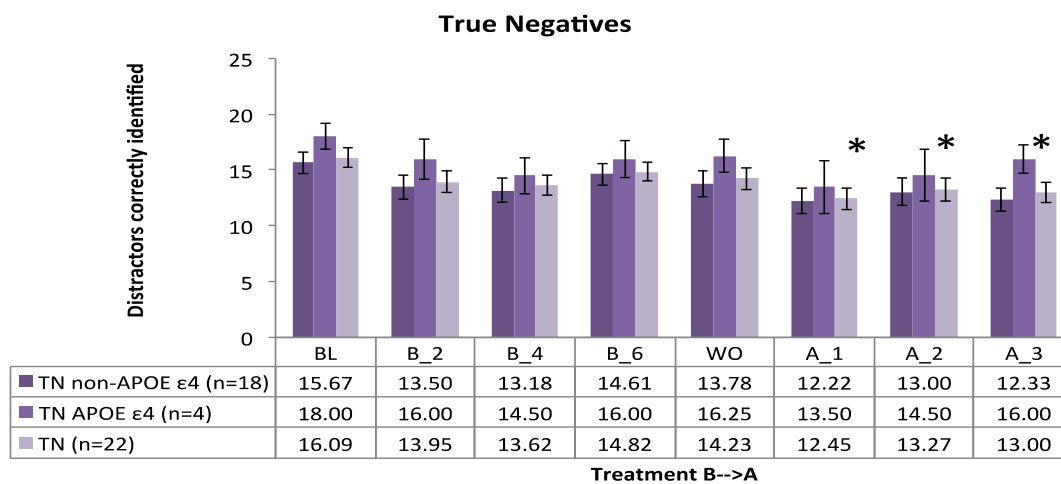
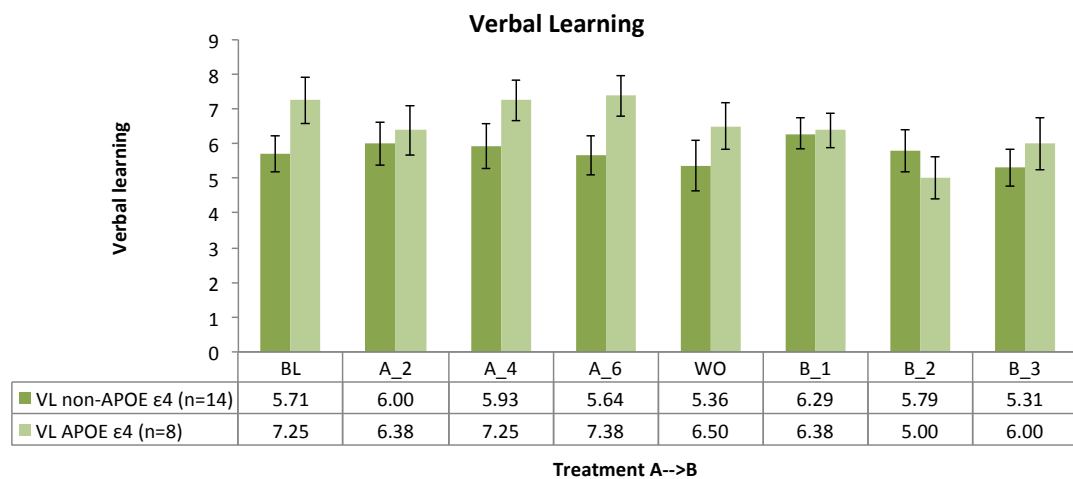


Figure 7.9 : Distractors Correctly Identified (True negatives) score for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. There were significant decrease of true negatives score (as measured by RAVLT) observed in all of the groups in treatment A→B (Graph A) and following testosterone treatment in treatment B→A, regardless of APOE (Graph B). * $p < 0.05$ in comparison to baseline. Participants in both arms have better recognition memory over time, maybe due to the practice effect. No significant differences were observed between non-APOE ε4 and APOE ε4 group.

(A)



(B)

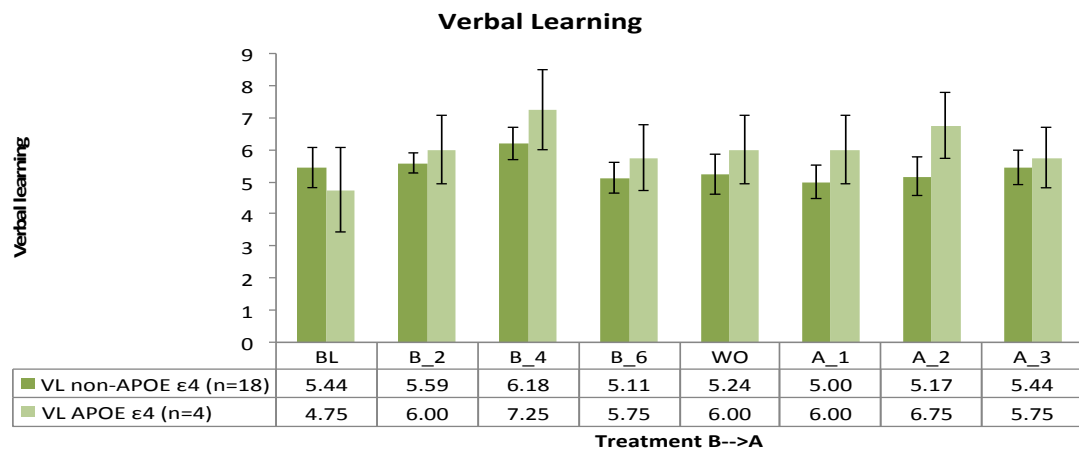
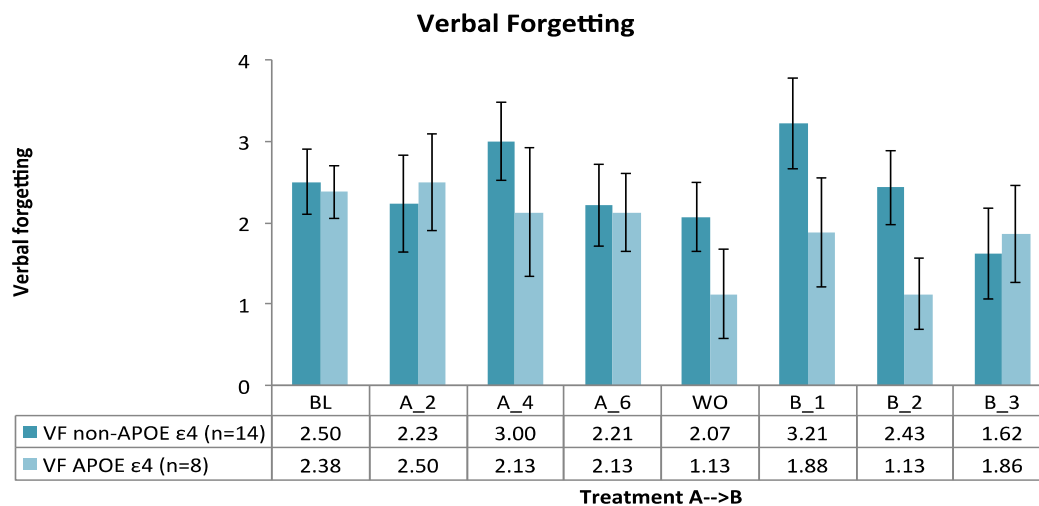


Figure 7.10: Verbal Learning score for treatments A→B (A) and B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (A) & B_2 to B_6 (B) = First Treatment period , WO=Wash-out, B_1 to B_3 (A) & A_1 to A_3 (B) = Second (Cross-over) Treatment. Bars represent mean \pm standard deviation, with mean values underneath the bars of each group. There were no significant differences in Verbal Learning (as measured by RAVLT) observed between any of the groups of either arm. There were significant differences between non-APOE $\epsilon 4$ and APOE $\epsilon 4$ groups, such that APOE $\epsilon 4$ participants had higher verbal learning scores. Verbal learning = Trial 5 – Trial 1 score (Sodergard, 1987).

(A)



(B)

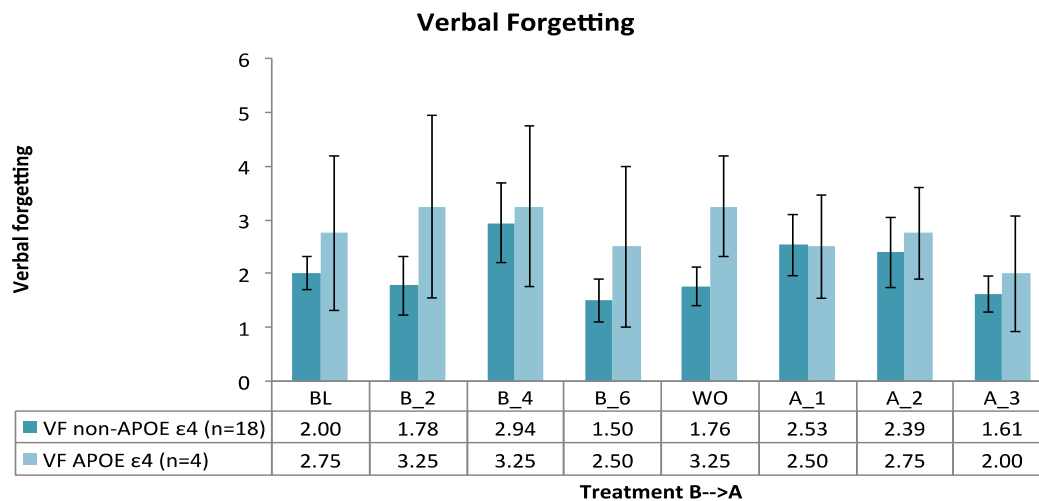
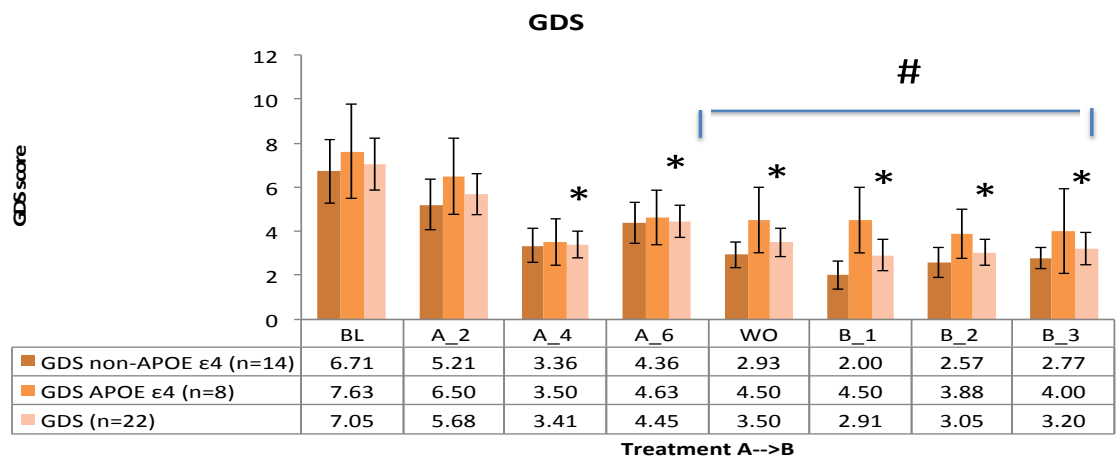


Figure 7.11: Verbal Forgetting (VF) score for treatments A→B (A) and B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (A) & B_2 to B_6 (B) = First Treatment period , WO=Wash-out, B_1 to B_3 (A) & A_1 to A_3 (B) = Second (Cross-over)Treatment. Verbal forgetting (loss of information) = Trial 6 – Trial 5 score⁴⁵ (recall after interference). Bars represent mean \pm standard deviation, with mean values underneath the bars of each group. There were no significant differences observed between any of the groups of either arm. However, a trend towards a decrease in verbal forgetting was observed following testosterone treatment. No significant differences were observed between non-APOE ϵ 4 and APOE ϵ 4 groups.

(A)



(B)

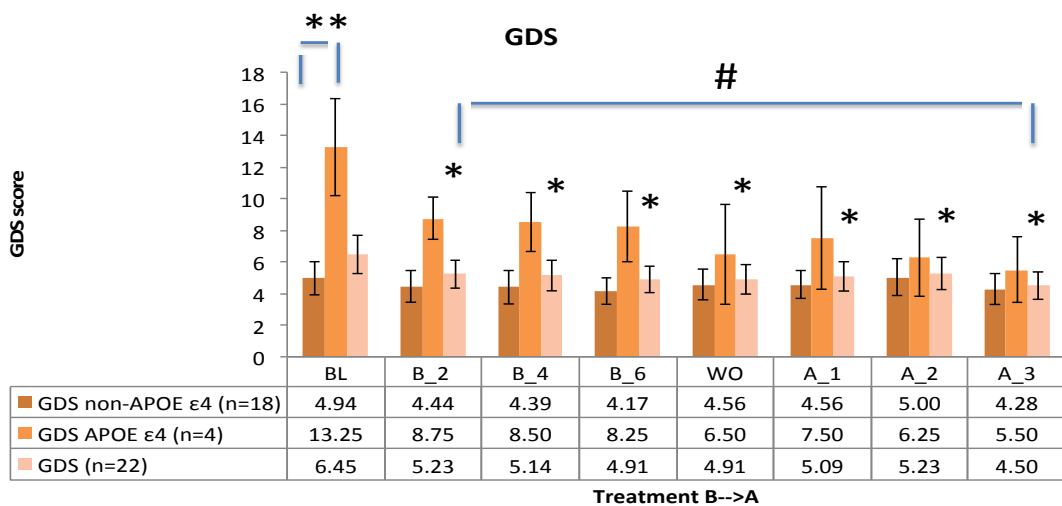
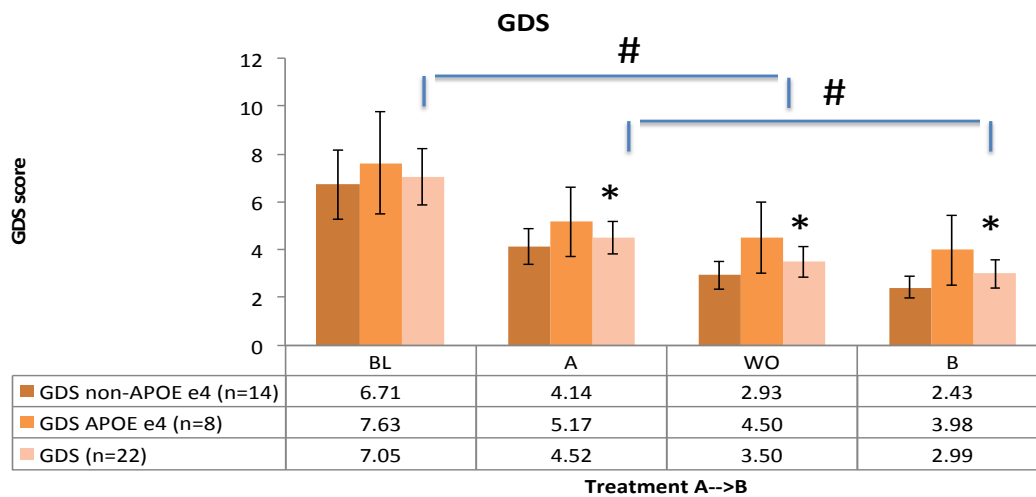


Figure 7.12 (a) : Depression score for treatment A→B (A) and treatment B→A (B) at 8 different time points. BL= Baseline, A_2 to A_6 (Graph A) & B_2 to B_6 (Graph B) = First Treatment period , WO=Wash-out, B_1 to B_3 (graph A) & A_1 to A_3 (Graph B) = Second Treatment or Cross-over period. Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. Depression score (as measured by GDS) was significantly decreased following testosterone treatment, washout, and following placebo treatment in both arms, regardless of APOE (Graph A & B). $p < 0.05$ in comparison to baseline of each arm of the treatments. # $p < 0.05$ in comparison between groups. Significant difference was observed between non-APOE ε4 and APOE ε4 group at baseline in treatment B→A only (** $p < 0.05$)

(A)



(B)

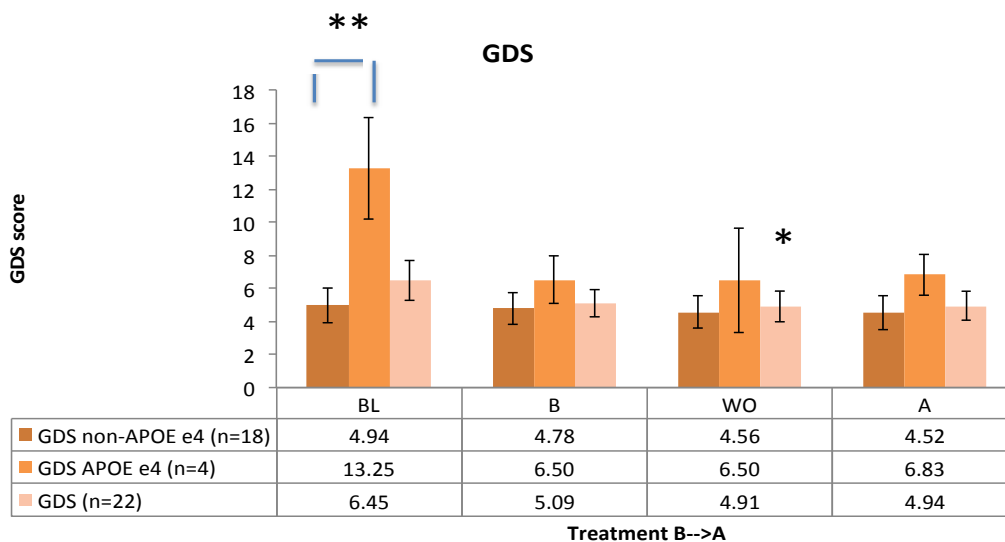


Figure 7.12 (b) : Depression score for treatment A→B (A) and treatment B→A (B) at 4 different time points. BL= Baseline, B = Mean of Testosterone treatment , WO=Wash-out, B = Mean of Placebo treatment. Bars represent mean and standard deviation (+), with values underneath the bars for mean of each group. Depression score (as measured by GDS) was improved by testosterone treatment and carry-over effect was observed in treatment A→B, regardless of APOE (Graph A)* $p < 0.05$ in comparison to baseline of each arm of the treatments. # $p < 0.05$ in comparison between groups. Significant difference was observed between non-APOE ε4 and APOE ε4 group at baseline in treatment B→A only.

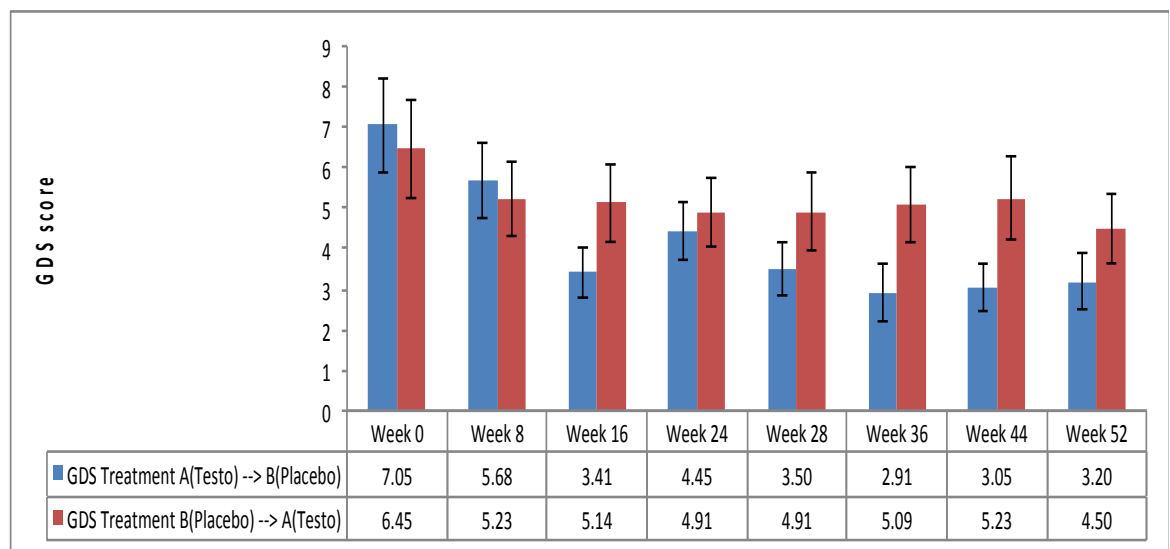
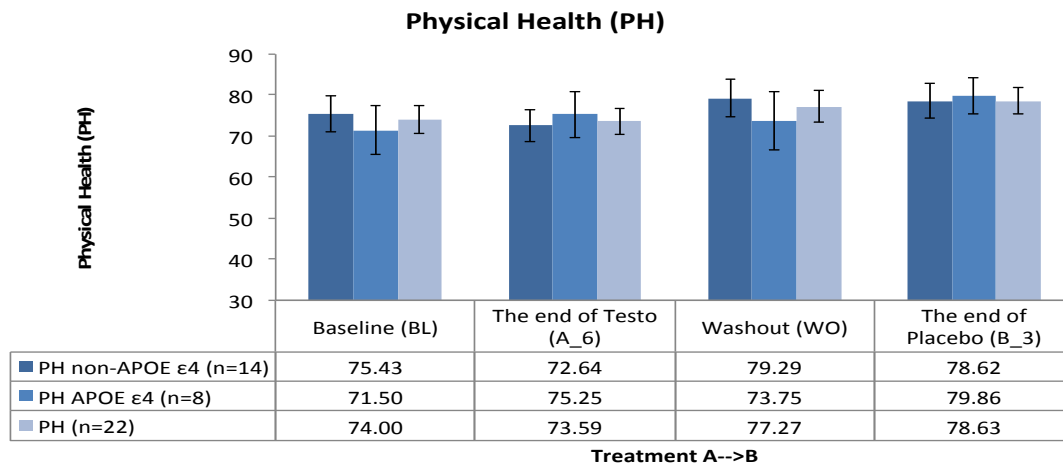


Figure 7.13: GDS score comparison between treatment arms A→B and B→A. Bars represent mean \pm standard deviation, with values underneath bars being the mean of each group. No significant differences were observed between any of the groups of either arm. N=22 for each arm.

(A)



(B)

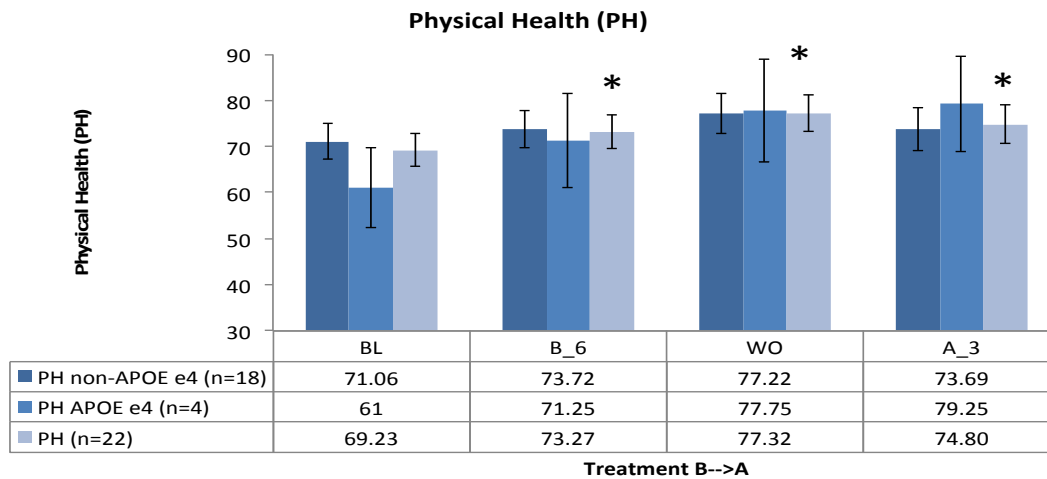
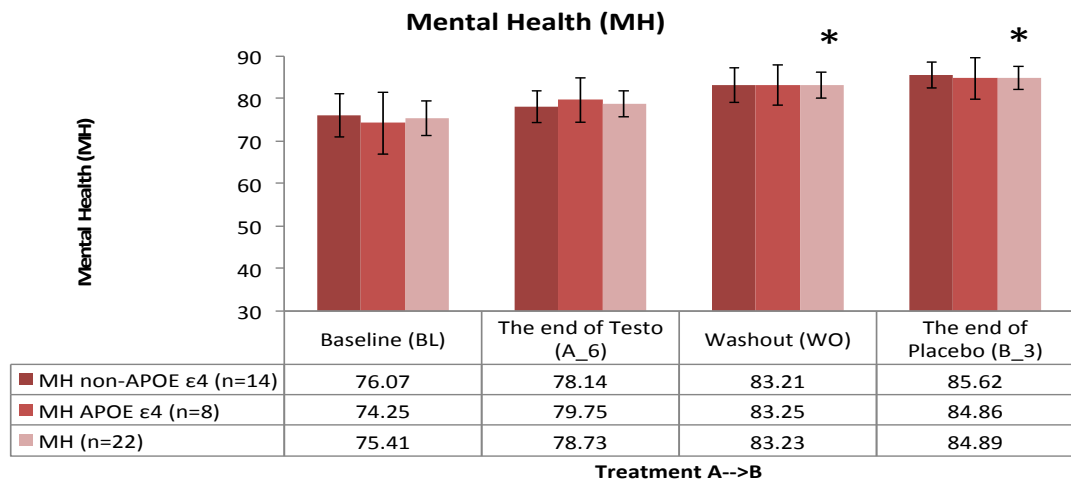


Figure 7.14 : Physical health (PH) score for treatment A→B (A) and treatment B→A (B) at 4 different time points. BL= Baseline (Week 0), A_6 & B_6 = End of First Treatment period (Week 24), WO=Wash-out (Week 28), B_3 & A_3 = End of Second Treatment or Cross-over period (Week 52). Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. There were no significant differences of PH score (as measured by SF-36) observed in treatment A→B (Graph A). However, PH score were significantly increased starting after placebo treatment, washout, and after testosterone in treatment B→A, regardless of APOE (Graph B). * $p < 0.05$ in comparison to baseline of each arm of the treatments. No significant differences were observed between non-APOE ε4 and APOE ε4 group.

(A)



(B)

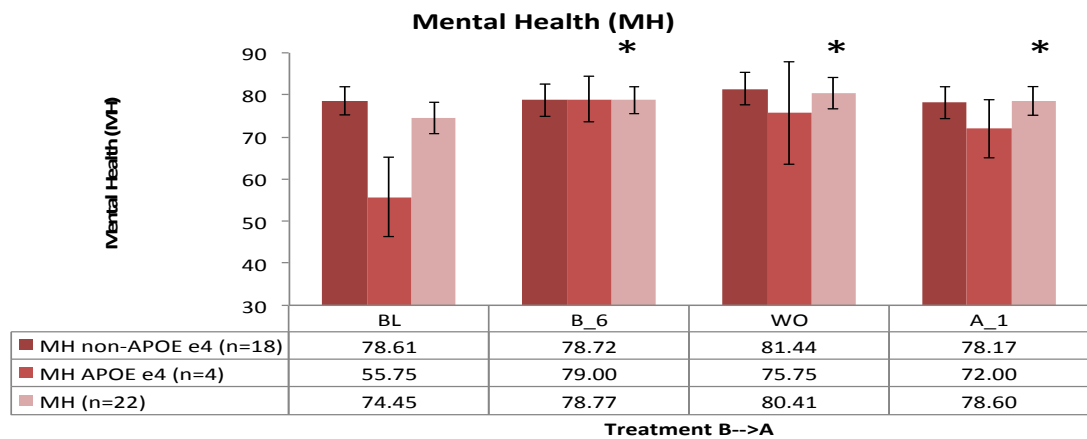
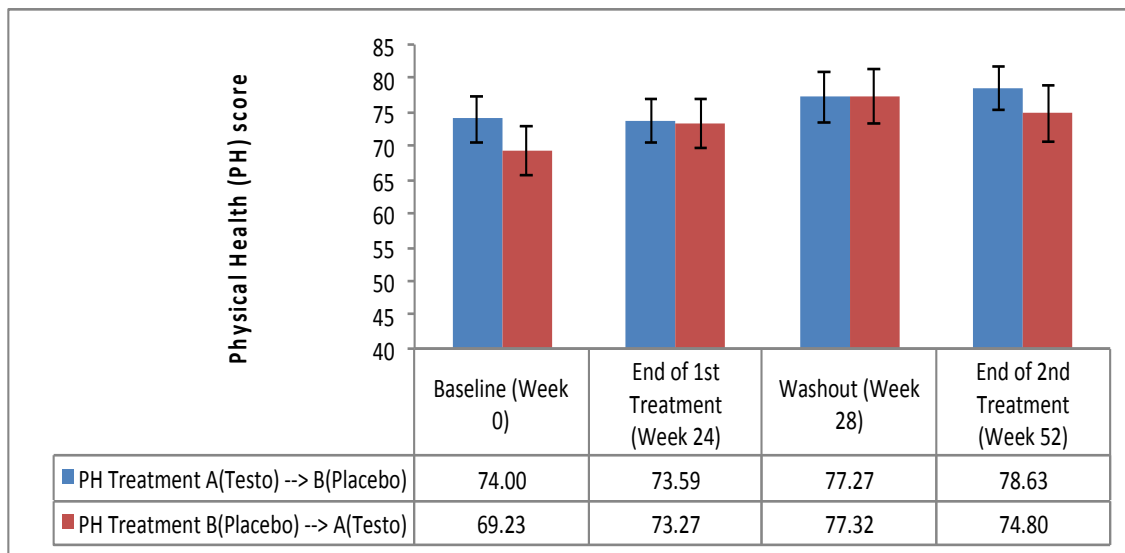


Figure 7.15 : Mental health (MH) score for treatment A→B (A) and treatment B→A (B) at 4 different time points. BL= Baseline (Week 0), A_6 & B_6 = End of First Treatment period (Week 24), WO=Wash-out (Week 28), B_3 & A_3 = End of Second Treatment or Cross-over period (Week 52). Bars represent mean and standard deviation (\pm), with values underneath the bars for mean of each group. MH score significantly increased at washout and after placebo in treatment A→B (Graph A). Whilst in treatment B→A, significant increased of PH score were observed starting after placebo treatment, washout, and after testosterone, regardless of APOE. * $p < 0.05$ in comparison to baseline of each arm of the treatments. # $p < 0.05$ in comparison between groups. No significant difference was observed between non-APOE ε-4 and APOE ε4 group.

(A)



(B)

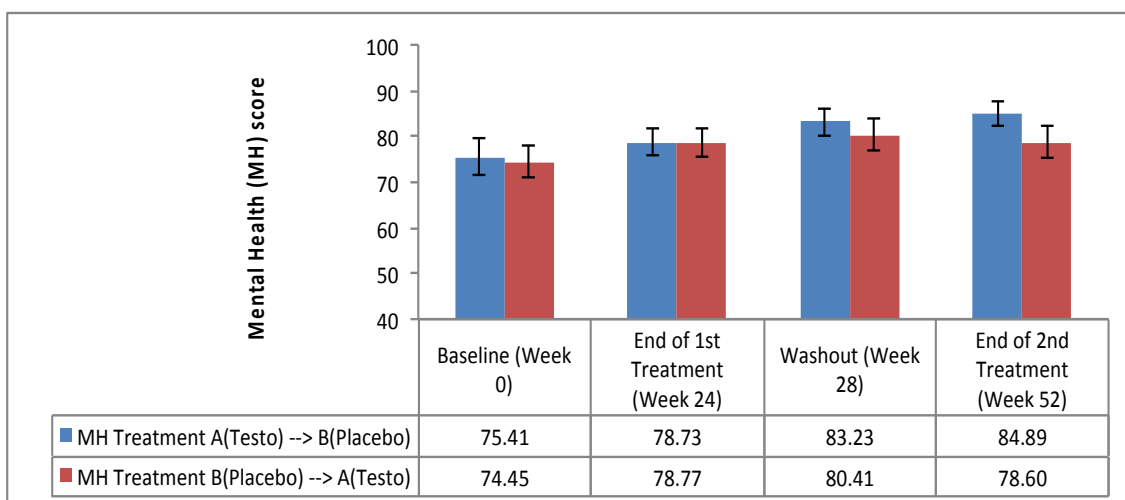


Figure 7.16 Physical Health score (A) and Mental Health score (B) comparison between treatment arms A→B and B→A. Values are presented as mean \pm standard deviation. No significant differences were observed between any of the groups of either arm . N=22 for each arm.

7.5 Discussion

Upon examining the relationship between circulating levels of testosterone and cognition in older men, it is important to note that only small amounts of total plasma testosterone (+2%) may pass the blood-brain barrier and enter the brain (Sodergard, 1987). Given that lower levels of testosterone are associated with cognitive impairment, testosterone administration might have a beneficial effect. Although studies examining the association between testosterone levels and cognition have frequently used measures of free testosterone, in this study total testosterone was measured as this has previously been shown to be a sensitive and valid measure for hormone treatment studies. Indeed, our study has shown significant improvements on MMSE scores after testosterone treatment in both arms of the study compared to baseline (Figures 7.1 A and B). Interestingly, in treatment A→B significant effects were also observed in the washout and placebo treatments. These results suggest that carry-over effects from the testosterone treatment lasted at least 28 weeks post-testosterone treatment. These apparent beneficial effects of testosterone on general cognition are in line with results obtained in a previous study by Slabbekoorn *et al.* (1999) which indicated that changes in cognition from hormone manipulation do not return to baseline levels but remain or continue to change in the same direction after cessation of hormone treatment. Therefore, carry-over effects of testosterone treatment delay further memory impairment.

In addition, another study by Cherrier *et al.* (2001) found that testosterone supplementation in elderly men improved visuospatial abilities as well as verbal memory even in the short-term period of 6-weeks. It remains unclear whether the improvements in cognition are due to increased testosterone levels, or estradiol levels, or both. Beneficial effects of testosterone treatment have also been observed by Muller *et al.* (2005) who demonstrated a positive relationship between overall cognitive functioning and free testosterone levels in older men.

Verbal memory has been measured in a variety of standardized episodic memory measures. RAVLT was chosen in this study because of its ease of administration and its sensitivity to impairment in different memory components (Dal Forno *et al.*, 2005). Furthermore, the RAVLT is a very efficient instrument for assessing episodic verbal

memory, which allows the evaluation of acquisition and recall of information (Mitrushina *et al.*, 2005). Because of possible rapid effects of testosterone, RAVLT were administered once every 2 months to monitor changes in verbal memory. As mentioned before, global cognitive changes as measured by MMSE were improved following testosterone treatment in this study. However, verbal memory and recall was not significantly improved following testosterone supplementation as measured by RAVLT (Tables 7.1 – 7.4, and Figures 7.3 – 7.7 and 7.11). This is in line with findings from the Hogervorst *et al.* (2004) study which showed that some neuropsychological tests could be more sensitive to hormone levels than others. Our RAVLT data (Fig 7.10 and Learning curve in Appendices) show that the increasing number of words recalled were more likely due to the improvement of memory over time because of practice or learning effects, rather than testosterone treatment itself. In addition, there may be a psychological effect of using the cream which we could not control for. That is, the participants strived to do their best in each tests (placebo effects) to receive the treatment. Therefore, it is quite difficult to differentiate between testosterone and placebo effects in terms of increasing verbal memory and words recall over time in this study. These results are in accordance with a study by Emmelet-Vonk *et al.* (2008), which studied men aged 60-80 years old with testosterone levels < 13.7 nmol/L. Their cognitive levels were measured by the same word lists of RAVLT in each treatment group. Other studies by Wolf and Kirschbaum (2002) and Yonker *et al.* (2006) further indicate non-significant associations between testosterone and episodic memory.

Most studies of testosterone treatment in testosterone deficient men have found no effects on cognition. It has been hypothesized that this could be due to decreased sensitivity of androgen receptors after prolonged exposure to low testosterone conditions (Hogervorst *et al.*, 2005). Alternatively, Cherrier *et al.* (2007) observed memory performance in 57 healthy elderly men after replacing testosterone with 50, 100, or 300 mg weekly injections. They found that only men with moderate testosterone increases demonstrated verbal and spatial memory improvement whilst men with low or high increases did not. The total testosterone levels shown in this study (Chapter 6) have shown high increases following testosterone treatment. Therefore, improvements in verbal memory following this testosterone treatment were subtle. This may indicate an ideal range of testosterone with respect to verbal memory improvement.

Another study by Wolf *et al.* (2002) also suggests that testosterone has no effect on verbal memory and is negatively associated with verbal fluency in men. Although this study did not explain the negative associations, the results are in line with those of another study which investigated the effects of long-term testosterone replacement on several neuropsychological tests (Sih *et al.*, 1997), and also found negative associations

Initially, this study used four different types of RAVLT at the visits to minimize learning effects which could overshadow any treatment effects. However, the use of different types of RAVLT in this study may have reduced the sensitivity in detecting any subtle testosterone treatment effects on verbal memory.

Depression affects a large number of elderly individuals and has been associated with cognitive decline (Yaffe *et al.*, 1999) and increased risk of mild cognitive impairment (MCI) (Geda *et al.*, 2006). Depression is also a common feature of testosterone deficiency and it usually responds well to testosterone treatment (Gooren, 2007). With testosterone levels in the hypogonadal range, depression was a common occurrence (Zitzmann *et al.*, 2006). Assessments may have been complicated by this fact as performance in cognitive tests can be influenced by depression (Yeap *et al.*, 2008). The results of these experiments show that participants with APOE $\epsilon 4$ alleles tend to have higher depression scores when compared to non-APOE $\epsilon 4$ carriers (Fig 7.12 A&B). Interestingly, the depression score of participants with APOE $\epsilon 4$ (n=4) in treatment B \rightarrow A was significantly different than the non-APOE $\epsilon 4$ at baseline, even they have been categorized to be depressed based on GDS cut-off score (Fig 7.12 B). Overall, as it was expected, depression was shown to be significantly reduced following testosterone treatment in both treatment arms (Fig 7.12 A&B), although in treatment B \rightarrow A the effect was only seen in APOE $\epsilon 4$ carriers, and may not have been due to treatment as GDS scores dropped initially in the placebo treatment time, which preceded testosterone treatment. The results for the APOE $\epsilon 4$ carriers more likely reflects the low number of participants (n=4). The baseline already-low GDS scores (mean = 5) in treatment B \rightarrow A for the non- APOE $\epsilon 4$ carriers (unlike the higher baseline GDS scores in the A \rightarrow B treatment group, means of 7 and 8) makes it rather difficult to determine a drop in depression using this scoring system. Interestingly, in arm treatment A \rightarrow B, the testosterone effect was carried over to the washout and placebo treatment, with further reductions in depression levels (Fig 7.12 A). The significant reduction in GDS score

with placebo treatment observed for the APOE ϵ 4 carriers in treatment B \rightarrow A (Fig 7.12 B). may be due to the baseline condition of most participants. The APOE ϵ 4 carriers had high depression levels at baseline, giving the treatment greater opportunity to improve GDS scores following treatment. As mentioned earlier though, the significant reduction of depression followed placebo treatment, which together with the large error bars on the graphs, more likely reflects the low number of participants. The results of treatment A \rightarrow B however are in agreement with Seidman *et al.*, 2001 who reported that total testosterone levels are negatively associated with depression in middle-aged men.

The association between depression and AD is still unclear. Associations are complicated by the possibility that depression may be a prodromal symptom of AD, which may appear before the recognition of AD. As a result, there are still insufficient data to determine whether depression represents an early symptoms or an independent risk factor of AD (Jorm, 2001).

There are limited data on the effects of testosterone on the quality of life in testosterone deficient men. However the information that exists suggests that testosterone can improve the quality of life significantly (restoring to the same level as men with normal testosterone levels) and the more severe the symptoms before treatment, the greater the benefits of testosterone replacement (Moncada, 2006). Although the effect of testosterone deficiency on quality of life has not been well established, it is clear that testosterone deficiency in the elderly has a negative effect on physical, mental and social functioning (Novak *et al.*, 2002).

In this study, quality of life comprising physical health and mental health were not significantly improved by testosterone treatment. Although, in treatment B \rightarrow A, both physical and mental health scores were significantly increased after being treated with testosterone compared to baseline. However, it is important to note that there were “carry-over effect” observed in which physical health (PH) and mental health (MH) at washout were still significantly different from baseline (Figure 7.14 B and Figure 7.15 B). In accordance with our results, Reddy *et al.*, 2000 and Kenny *et al.*, 2002 also failed to show testosterone therapy-induced improvements in quality of life testosterone therapy. These two studies used the SF-36 test too. Using a different measure, namely the Quality of Life-Alzheimer Disease (QOL-AD), Lu *et al.* 2000 also observed no

significant difference following the assessment of quality of life in healthy elderly men treated with testosterone for 24 weeks.

However, in a study by Snyder *et al.*, 1999 which used the SF-36 test for assessment, transdermal testosterone treatment in men over 65 years of age improved the patients' perception of physical functioning (but no other domains of the SF-36) compared with placebo. This study observed the effect of testosterone treatment for 36 months, whilst the treatment in the current study lasted only 6 months. The testosterone replacement therapy by Kenny *et al.*, 2002 for 12 months also did not show improvement of quality of life. The effect of testosterone on quality of life in this study was not significant enough to be detected – this may be because the quality of life was determined to be quite adequate in these participants at baseline.

It is possible that the SF-36 assessment is too general and not particularly suitable for assessing quality of life in a testosterone deficient population (Jorm, 2001). There are other more specific scales to measure quality of life in testosterone deficient men, such as The Aging Male's Symptoms (AMS) (Daig *et al.*, 2003) and the Age-Related Hormone Deficiency-Dependent Quality of Life questionnaire (A-RHDQoL) (McMilan *et al.*, 2003). The questionnaires detailed in these studies may be more specific in addressing issues related to hormone deficiencies, and may assess better the effects of testosterone replacement therapy in men with low testosterone and show that quality of life can improve following testosterone treatment.

A study from my supervisor's laboratory (Sohrabi, *et al.*, 2009) has shown that mental health and quality of life in memory complainers were independent of APOE genotype. Results in the current study were consistent with these previous findings as they did not show significant differences between APOE ϵ 4 and non-APOE ϵ 4 carriers in relation to quality of life, in the A→B treatment arm, although there appears to be a trend towards better physical health in both APOE ϵ 4 and non-APOE ϵ 4 carriers at all stages following testosterone treatment (Fig.7.14). A similar trend is visible in the B→A group, however this is possibly due to the (only 4) APOE ϵ 4 carriers having a low baseline score, which is followed by the apparently high physical health scores at washout stage, which is obviously not due to the subsequent testosterone treatment in this group. These results show that either the number of APOE ϵ 4 carriers and/or the

size of the whole B→A cohort is too small for good statistical analysis, the SF36 test from which the physical health scores were obtained is not appropriate for assessment of testosterone treatment, or both. From the perspective of mental health scores, in the A→B group there appears to be a trend towards better mental health in both APOE ε4 and non-APOE ε4 carriers over the stages following testosterone treatment. In the B→A group, no change can be seen in the non-APOE ε4 carriers (Fig. 7.15), however due to the low baseline score of the APOE ε4 carriers, it appears that the placebo treatment has raised mental health levels, which then stay high for the rest of the trial. This discrepancy, again, may be due to the very small sample sizes for APOE ε4 carriers compared to non-APOE ε4 rather than any effect of possession of APOE ε4 alleles.

Controlled clinical trials of testosterone supplementation in healthy elderly men with partial androgen deficiency have yielded inconsistent results in relation to quality of life. Some studies have found an increased sense of well-being (Tenover, 1992 ; Park *et al.*, 2003) while others have observed no significant effects (Reddy *et al.*, 2000). Assessment of quality of life offers a window of opportunity into the effects of testosterone intervention on the patients and caregivers' perceived well-being and may assist clinicians in determining the potential functional benefits and risks of therapy.

This study had some limitations. This study was underpowered, with relatively small sample sizes (n=44) to find associations between testosterone treatment and cognition, despite its cross-over design. There may be age dependent optimal testosterone levels which vary for different brain functions which we did not investigate in this study. Furthermore, the chosen instrument of RAVLT may lack sensitivity to detect small or subtle changes in cognitively intact participants within 6 months of testosterone treatment. Therefore, longer duration of studies may have yielded greater effects of testosterone on cognition and verbal memory (Yonker *et al.*, 2006 ; Gordon *et al.*, 1986). It is also important to note that comparisons with other studies are difficult since there were large variations in testosterone levels across studies despite having similar age ranges. Furthermore, many studies have used different cognitive tests to assess similar brain functions, and sometimes have used insensitive tests. Another potential explanation for differences in results is that many studies have measured total testosterone (Hassler *et al.*, 1992 ; Gordon, 1986 ; Tan, 2005) rather than free testosterone (Hooven *et al.*, 2004 ; Moffat, 1996 ; Gouchie, 1991 ; Christiansen, 1987).

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However, this study has attempted to measure general cognition (with verbal memory, in particular), depression level, and quality of life at various time points of treatment while capturing peak testosterone levels.

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Chapter 8

Effects of testosterone treatment

on brain metabolite levels

and on rates of medial temporal atrophy

8.1 Introduction

Alzheimer's Disease (AD) cognitive symptoms arise only after extensive synaptic loss and neuronal death has already occurred. As a result, diagnosis is usually made many years after the start of the disease process. A screening process that could provide early, accurate diagnosis of AD would enable earlier intervention, and thus allow current and future treatments to be more effective. Potential biomarkers for AD screening are being sought from many fields including genetic analysis, blood proteomics, cerebrospinal fluid proteomics and brain imaging. With respect to imaging techniques, both structural and functional neuroimaging methods are being tested.

AD is characterized by early loss of memory, with the hippocampus and entorhinal cortex being the first sites of pathological changes. Brain structural changes include generalized cerebral atrophy, ventricular enlargement and mild to moderate white matter changes or leukoaraiosis. Focal temporal atrophy can be detected using computerised tomography, and a suggested diagnostic marker for AD involves the use of angled temporal lobe views to detect medial temporal lobe atrophy associated with the disease (O'Brien, 2007). Unfortunately this has been found to have limited utility both in early disease and in the differentiation of AD from other causes of dementia. Magnetic resonance imaging (MRI) has been widely investigated in AD and consistent with the temporal development of plaque and NFT pathology, very early structural changes have been demonstrated in the hippocampus and entorhinal cortex. However, the particular structure that would be most useful for early diagnosis remains unclear (Scheltens *et al.*, 2002, Du *et al.*, 2001). Atrophy of the hippocampus and entorhinal cortex can be assessed using simple validated scales, and such atrophy occurs in 80-90% of AD cases and in around 5-10% of control subjects (Du *et al.*, 2001). Other early changes in AD include atrophy of the posterior cingulate (Scahill *et al.*, 2002), not a prominent site of pathology but changes may be related to loss of afferent input from associated fibres.

Serial MRI has been useful in increasing our understanding of disease progression in AD and other dementias. Atrophy rates of around 2% per year occur in AD compared to 0.25-0.5% in control subjects (O'Brien, 2007), and using serial MRI, it has been possible to identify structural changes in familial AD cases 2-3 years before they could

be detected by cross-sectional volumetric measurement (Ridha *et al.*, 2006). Serial MRI has sometimes been used as an outcome measure in clinical trials, being a robust biological measure that shows little variability. Power calculations have shown that clinical studies of disease progression can be undertaken with significantly fewer subjects when using serial MRI as opposed to a clinical outcome measure (Fox *et al.*, 2000). Using this method, it has been possible to measure a decreased rate of hippocampal atrophy in those receiving cholinesterase inhibitors (Hashimoto *et al.*, 2005). Serial MRI in another study measured increased brain atrophy in a group of patients given the active amyloid vaccine. This was suggested to be due to increased clearance of amyloid plaques from the brain (Fox *et al.*, 2005).

Atrophy of the medial temporal lobe (MTA), including the hippocampus and entorhinal cortex, is a sensitive marker for AD, and studies have shown that decreased volume of the medial temporal lobe is also marker for dementia in patients with mild cognitive impairment (MCI) (Esther *et al.*, 2004). However, MTA is present in other dementias as well, for example in frontotemporal lobe degeneration (FTLD), vascular dementia (VaD) and dementia with Lewy bodies (DLB). It may also be seen in normal aging. Volumetric assessment of MTA is difficult because of the time-consuming analysis required, and because automated hippocampal volume measurement techniques are not widely available. In contrast, the assessment of MTA using a standardized visual rating scale is a quick and easy measurement, with comparable predictive accuracy. Such visual assessment of MTA from brain MRI using a standardized rating scale is powerful and can function as an independent predictor of conversion to dementia in relatively young MCI patients (Scher *et al.*, 2007). In fact, the MTA scale has been shown to be a better predictor of memory impairment when compared to neuropsychological tests (Scheltens *et al.*, 2002). AD hippocampal volumes have been measured to be 11.5% smaller than in control cases, with similar size differences for the median left (12%) and median right (11.6%). Hippocampal shape analysis has shown that differences are more evident in the hippocampal body than in the head, and more consistent differences have been found in the left hippocampus than the right (Scher *et al.*, 2007, Kantarci *et al.*, 2004).

Functional MRI is another form of scanning that can measure brain activity indirectly, by measuring blood flow in the brain. Deoxygenated hemoglobin attenuates the MR

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signal, thus a vascular response leads to a signal increase that is related to neural activity. It has several potential advantages, particularly for clinical trials, as it is a non-invasive technique that does not require the injection of contrast agent or radiation exposure and thus can be repeated many times during a longitudinal study. fMRI has relatively high spatial and reasonable temporal resolution, and can be acquired in the same session as structural MRI (Sperling, 2011).

In an AD brain, volume loss has been interpreted to reflect neuron loss, possibly together with non-neural changes, such as loss of glial components. Such changes are now thought to be detectable using yet another relatively new technology - proton magnetic resonance spectroscopy (^1H MRS), which has the potential to provide non-invasive assessments of metabolic and functional correlates of dementia in research and clinical settings (Lin *et al.*, 2005). An MRS spectrum shows frequency or chemical shift (compared to a reference chemical) on the horizontal axis, which allows the identification of the metabolites being investigated, and the vertical axis shows the signal strength in arbitrary units. MRS allows for non-invasive and repeated quantitation of several different brain metabolites, most commonly N-acetyl aspartate (NAA), choline (Cho), myo-Inositol (mI), and creatinine (Cr) containing compounds.

Each of these metabolites is thought to be sensitive to a different aspect of *in vivo* pathologic processes at the molecular or cellular level (Kantarci *et al.*, 2007). Increases in choline and myo-Inositol (markers of membrane turnover) have been demonstrated in several studies of AD brains, implying significant cellular membrane (and glial) pathology. Large decreases in brain NAA (a marker of neuro-axonal integrity) are commonly seen in grey and white matter in AD (as well as in other forms of dementia), indicating the presence of significant axonal damage. Levels of NAA also appear to correlate with disease severity. Recent studies suggest MRS has the potential to distinguish AD patients from healthy control at pre-clinical stages of the disease in which AD-modifying therapies are likely to be most beneficial (Fallini *et al.*, 2005). For example, in a study of cognitively normal older adults, higher ratios of mI/Cr and Cho/Cr were associated with higher A β loads, as measured by PiB retention. Higher Cho/Cr was found to be associated with worse performance on domain-specific cognitive tests independent of A β load, suggesting that Cho/Cr elevation may also be dependent on other preclinical dementia pathologies characterized by Cho/Cr ratio

elevation such as Lewy body or ischemic vascular disease in addition to A β load (Kantarci *et al.*, 2011).

Another recent study carried out MRS on MCI patients to see if it was possible to predict conversion to dementia within the 2-year period after baseline assessment. The brain regions examined were the posteromedial bilateral parietal lobe and left medial occipital lobe. After the 2 year follow-up, 27 (38%) patients converted to AD. The mean NAA/Cr ratio in the poster medial bilateral parietal cortex was 1.38 in converters versus 1.49 in non-converters ($p < 0.0001$). In this study, neither the APOE $\epsilon 4$ genotype nor leucoaraiosis was predictive of conversion to dementia (Modrego *et al.*, 2011). Several other studies have looked at various concentrations or ratios of the 4 metabolites (NAA/Cr, Cho/Cr, mI/Cr and NAA/mI ratios), such as one study which found that the NAA/Cr ratio in the posterior cingulate gyri showed considerable potential for distinguishing amnesic MCI from normal elderly controls (Wang *et al.*, 2012), and another study which compared AD and subcortical ischemic vascular dementia (SIVD), which are known to have overlapping pathologies and risk factors, and found that the pattern of mI concentration changes differed between the two disorders; mI was increased in AD but not increased in SIVD (Shiino *et al.*, 2012). All these results suggest MRS may provide a valuable biomarker for predicting the development of AD as well as to differentiate between AD, vascular dementia and MCI.

Another recent study showed that the discrimination between early AD and elderly healthy control subjects can be improved by adding MRS measures to MRI measures: it was found that combining MRI and MRS measures resulted in a sensitivity of 97% and a specificity of 94% compared to using MRI or MRS measures alone (sensitivity: 87%, 76%, specificity: 86%, 83% respectively) (Westman *et al.*, 2011). In AD patients, ^1H MRS measurements of NAA/Cr and mI/Cr ratios correlated with the neuropsychological measures of cognitive function. This correlation was found to be region-specific, depending on the cognitive domain being studied. For example, ^1H MRS metabolite changes in patients with AD correlate with verbal memory loss in the left medial temporal lobe, and correlate with language impairment and visuo-constructional abilities in the left parietotemporal cortex (Kantarci *et al.*, 2007).

Since a variety of metabolite differences have been recorded, possibly due to the region-specific nature of these differences, more studies need to be conducted to characterise these MRS differences further, and to discover which metabolite differences and which brain regions are the most appropriate for differentiating people with early stages of AD from healthy elderly controls.

With regard to the monitoring of treatment effects, there are very few studies that have directly examined testosterone's effects on brain activity (Moffat *et al.*, 2004). Applying this new MRS technology to measure brain metabolite changes in a testosterone treatment trial may indicate how testosterone is affecting brain function, thus providing valuable information. Overall, this chapter will investigate the effects of testosterone on brain metabolite changes and atrophy rates, as well as the association between MTA scale measurements and neuropsychological performance.

8.2 Aims

The aims of my study were:

- To determine whether testosterone treatment can influence brain metabolite levels in testosterone deficient men;
- To determine whether testosterone treatment alters rates of medial temporal lobe atrophy;
- To determine whether testosterone treatment in men influences hippocampal atrophy, as assessed by the MTA scale;
- To determine whether there is any correlation between MTA scale measurements and neuropsychological performance following testosterone treatment in testosterone deficient men.

8.3 Methods

Participants

All participants were randomized in both arms of the treatment, the details of which have previously been described in section 6.3. Testosterone deficient men were seen at baseline and underwent structural brain MRI and MRS, following completion of the neuropsychological assessment. Participants attended follow-up MRI and proton MRS imaging at the following intervals from baseline: at 24 weeks which was the endpoint of the first treatment (either treatment A or B), and at 52 weeks which was the end of the second treatment (either treatment B or A) respectively.

¹H MR Spectroscopy- Chemical Shift Imaging (CSI)

¹H MR Spectroscopy (MRS) spectra were acquired on a 1.5 T Philips Gyroscan ACS-NT system using a quadrature Head coil. MRI of the brain was done before the commencement of MRS for assessment of brain condition at that time. The first step of the MRI was a SCOUT / SURVEY. Twenty axial or transversal scout images were obtained by T2/FLAIR (Fluid Attenuated Inverse Recovery) using a thickness of 5 mm and 0.5 mm gaps. From these sequences, evaluation of the whole brain for lacunar infarcts or lesions was possible. Twenty sagittal scout images were then acquired by T1/FFE (Fast Field Echo) using a 5 mm thickness and 0.5 mm gap. This sequence represents the long axis of the hippocampus for coronal slice determination by MRS. The next acquisitions were 20 coronal scout images with T2/TSE (Turbo Spin Echo) at a thickness of 5 mm and gaps of 0.5 mm. Slices were made perpendicular to the long axis of the hippocampus body. For serial acquisition, the same voxel size was used for each patient in coronal slides and repositioned as accurately as possible by the same study radiographer who was blinded to clinical / cognitive information.

2D Spectroscopic Imaging (SI) methods (TR = 1500 ms, TE = 272, matrix = 512 x 512) were used for spectra generation. An Echo Time (TE) of 272 ms and water suppression techniques were used to reduce the contribution from underlying broad resonances. The

plane of the 2D SI grid can be angulated with the echo defined volume, the direction can be freely chosen to be transverse, sagittal or coronal so 2D SI could be use to capture an irregular shape structure in whole voxel. Five spectroscopic points were generated on the right and left hippocampi, right and left medial temporal lobe, and one in the white matter of the thalamus, this last one providing a “normal” spectrum which served as a control (no pathologies observed). Acquired spectra were reviewed visually by an experienced neuro-radiologist for quality as well as artifacts that would interfere with metabolite quantization. The metabolite ratios NAA/Cr and Cho/Cr were generated from the software of the Gyroscan ACS-NT system, using the physiologically stable metabolite Creatine (Cr) as the internal reference metabolite. Due to the selection of long echo time in this study, myo-Inositol was not available. A total of 1980 spectra were acquired. Total scan time for MRI and MRS acquisition was 50-60 minutes for each patient.

Medial Temporal Atrophy (MTA) rating

A standardized Scheltens scale was used to assess atrophy of the right and left medial temporal lobes including the hippocampus from hard film copies of coronal slice T1-weighted images, with slice thicknesses being 8 mm. Atrophy is graded on a 5-point scale (0=absent, 1=minimal, 2=mild, 3=moderate and 4=severe) based on the height of the hippocampus, the width of the choroid fissure, and the width of the horn. (Figure 8.1). These MTA ratings were performed at three time points, which were at baseline (before any treatment), the end of testosterone treatment, and the end of placebo treatment.

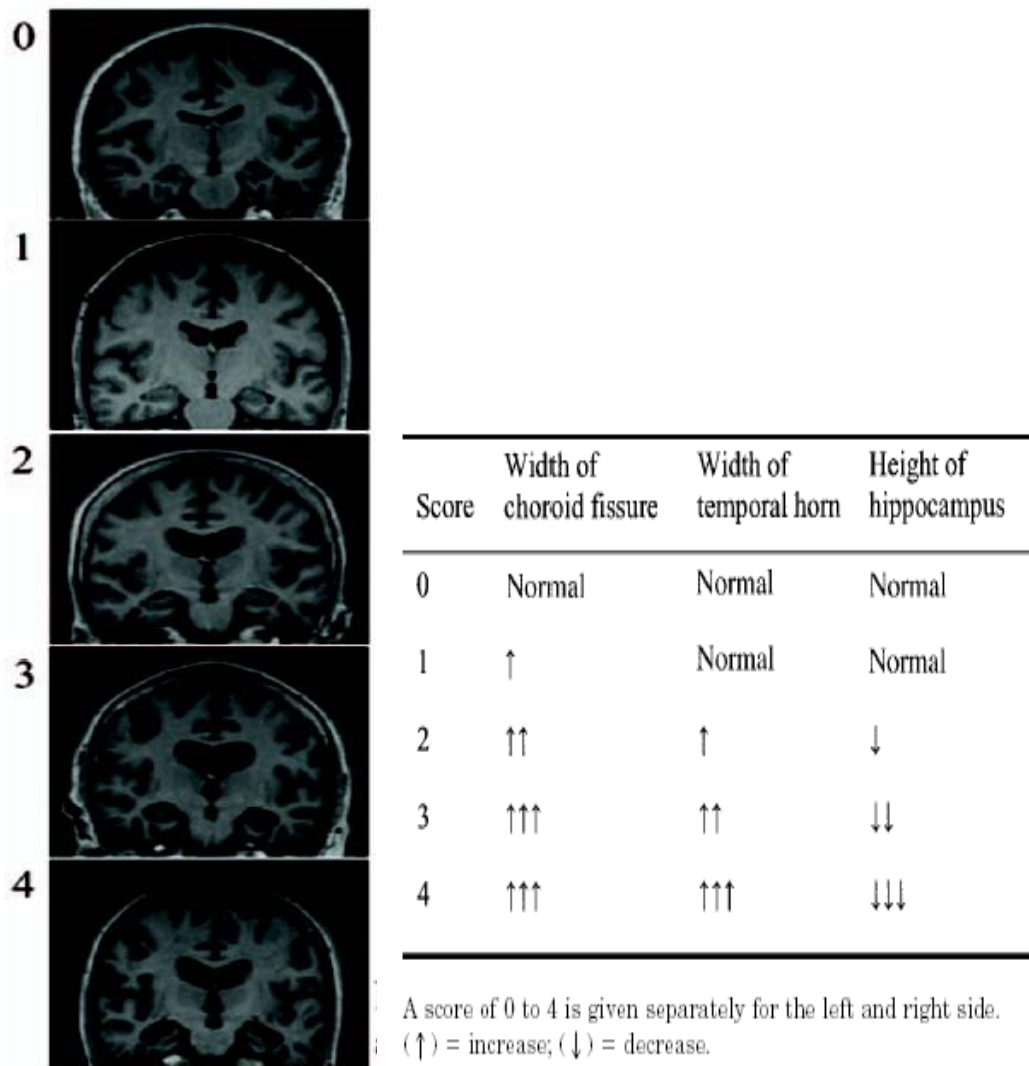


Figure 8.1: Medial Temporal Atrophy (MTA) visual assessment scale. MTA scaling performed on T1-MR coronal slices. The right and left sides are rated separately and also combined. Score range of atrophy: 0=absent, 1=minimal, 2=mild, 3=moderate and 4=severe.

For the purpose of statistical analysis, two experienced radiologists (blinded to treatments and age) were employed to provide two sets of results to assess atrophy in the right and left medial temporal lobes. In addition, the right and left MTA scales were combined to generate an average MTA score.

Statistical analysis

The Statistical Package of Social Sciences (SPSS version 19, SPSS Inc, Chicago, USA) was used for data analysis. The Shapiro-Wilk test was used to examine normality of distribution of continuous variables for each group. All of the data were found to be not normally distributed (non-parametric data), therefore Friedman's Test and/or Wilcoxon signed-rank test was used to look at differences between groups. The inter-class correlation coefficient was used to assess the inter-rater reliability of the qualitative MTA scale using Kappa Measure Agreement. Regression analysis was done in order to determine whether the efficacy of testosterone treatment in men can be determined by the rate of hippocampal atrophy. Partial correlations between MTA scores and measures of neuropsychological assessment (Mini Mental State Examination, Geriatric Depression Scale, and Rey Auditory Verbal Learning Test) were examined using Spearman Rho correlation, controlling for the effect of age and education.

Measures

The details about the neuropsychological, and clinical measures used in this study can be found in Chapter 7, page 267. Briefly, to measure the general cognitive functions and the potential cognitive decline severity Mini Mental State Examination (MMSE) and Geriatric Depression Scale (GDS) were used. The verbal memory was assessed using the Rey Verbal Learning Test (RAVLT).

8.4 Results

8.4.1 Effects of testosterone on brain metabolites as measured by ¹H MR Spectroscopy

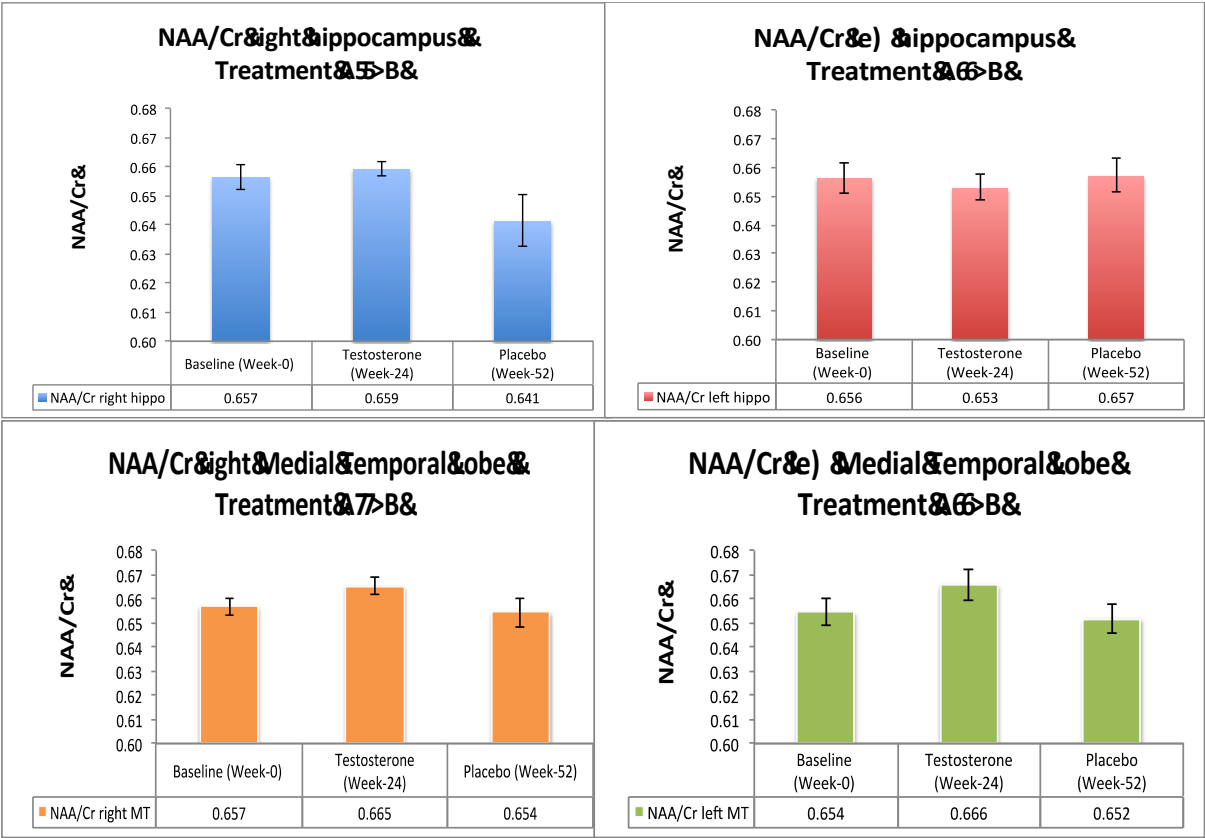
To determine the effects of testosterone on brain metabolite levels, the N-Acetyl-Aspartate, Choline (Cho) and Creatinine (Cr) levels were assessed using ¹H MRS and the NAA/Cr and Cho/Cr ratios were calculated. For an individual metabolite, the parts per million (ppm) refer to the unit of measure used to identify a metabolite's peak location or resonance on the x-axis. The ppm is calculated by dividing the difference in frequency (in Hertz) of two peaks (with one peak being the reference) by the operating frequency of the MR scanner (in Hertz). The signal intensity (amplitude on the y-axis) and line width provide the "area" which can be used to quantify the amount of the observed metabolites (Figure 8.2). Every metabolite has a normal concentration that generates a pattern of peaks which is uniform from person to person unless there is an underlying pathology. Therefore, diagnosis with MRS can be made by comparing between normal and abnormal peak patterns. According to numerical peak-height ratios with the assumption that Cr=1, a ratio of NAA/Cr of 1.25 ± 0.07 would suggest a dementia diagnosis. A value of NAA/Cr >1.11 may be classified as probable AD (Shonk *et al.*, 1995).



Figure 8.2: Example of a ^1H MRS-2DCSI set of scans with 5 different spectroscopic points obtained using a Gyroscan Intera 1.5T (Philips). 1: right hippocampus. 2: left hippocampus. 3: right medial temporal (MT) lobe 4: left MT lobe 5: thalamus (normal) area

The mean and standard deviation for longitudinal metabolite changes between testosterone and placebo treatment as revealed by NAA/Cr and Cho/Cr ratios are shown in Figures 8.3 and 8.4. There were no significant changes in the two ratios in either the left or right hippocampi, or the medial temporal lobes over the three time points (between baseline, treatment A, and treatment B) based on Friedman's test and Wilcoxon signed-rank test.

(A)



(B)

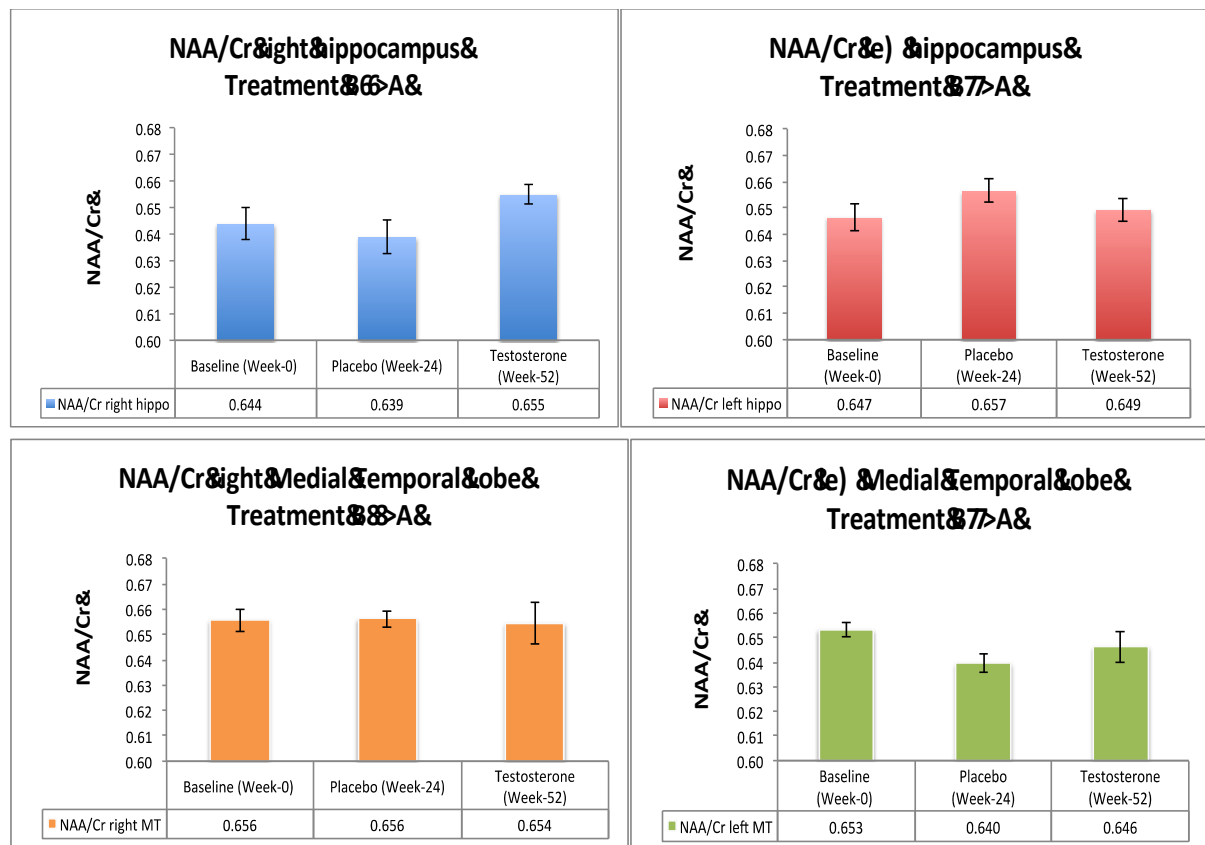
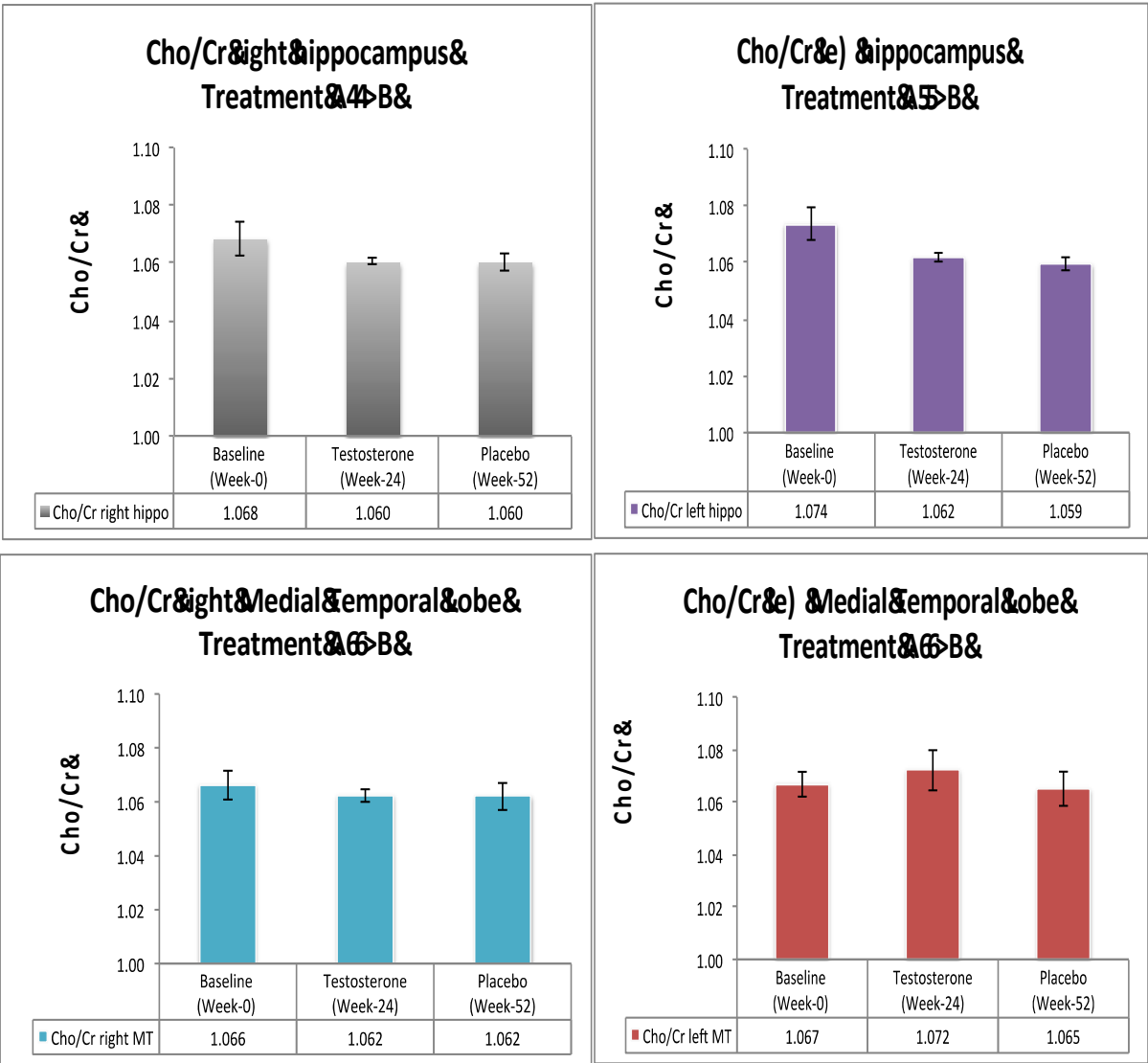


Figure 8.3 : NAA/Cr ratios measured at four different spectroscopic points in treatment A→B (A), and treatment B→A (B). There were no significant changes over time in any of the groups. (n=22).

(A)



(B)

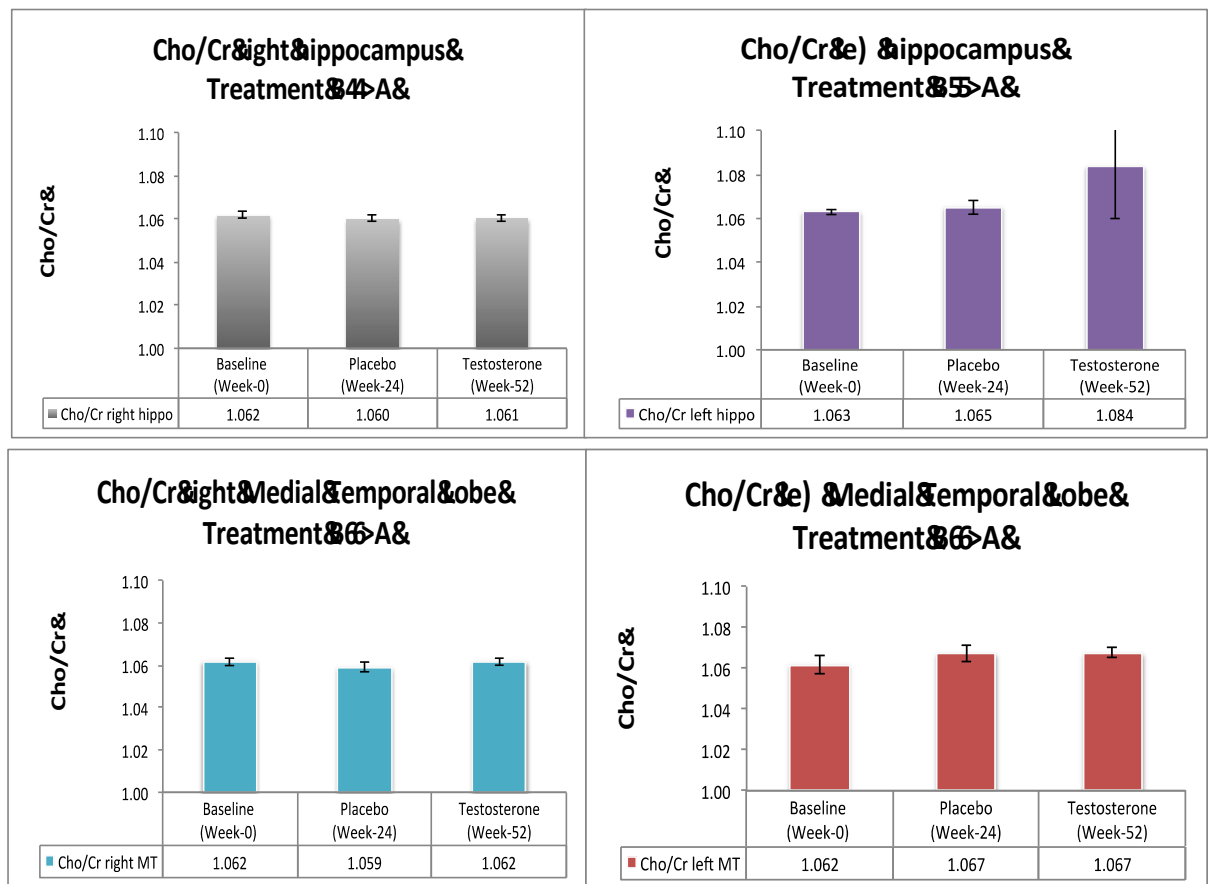


Figure 8.4 : NAA/Cr ratios measured at four different spectroscopic points in treatment A→B (A) and treatment B→A (B). There were no significant changes over time in any of the groups. (n=22).

8.4.2 Effects of testosterone on medial temporal atrophy (MTA)

The variability between the ratings put forward by the three different raters were investigated. There was little agreement with Kappa Measure of Agreement between raters 1 and 2 (0.01, 0.02), versus rater 3 (0.015). Therefore, the MTA scores were generated from two out of three radiologists whose scores were in close agreement.

MTA scale results were generated for the right and left temporal lobes separately, as well as combined, to generate one score which ranged from 0-4, with a greater score indicating more atrophy (as demonstrated in Fig. 8.1). The MTA scaling was done at three time points (as used in section above) in both arms of the experiment, with measurements taken at baseline, the end of testosterone treatment, and the end of placebo treatment.

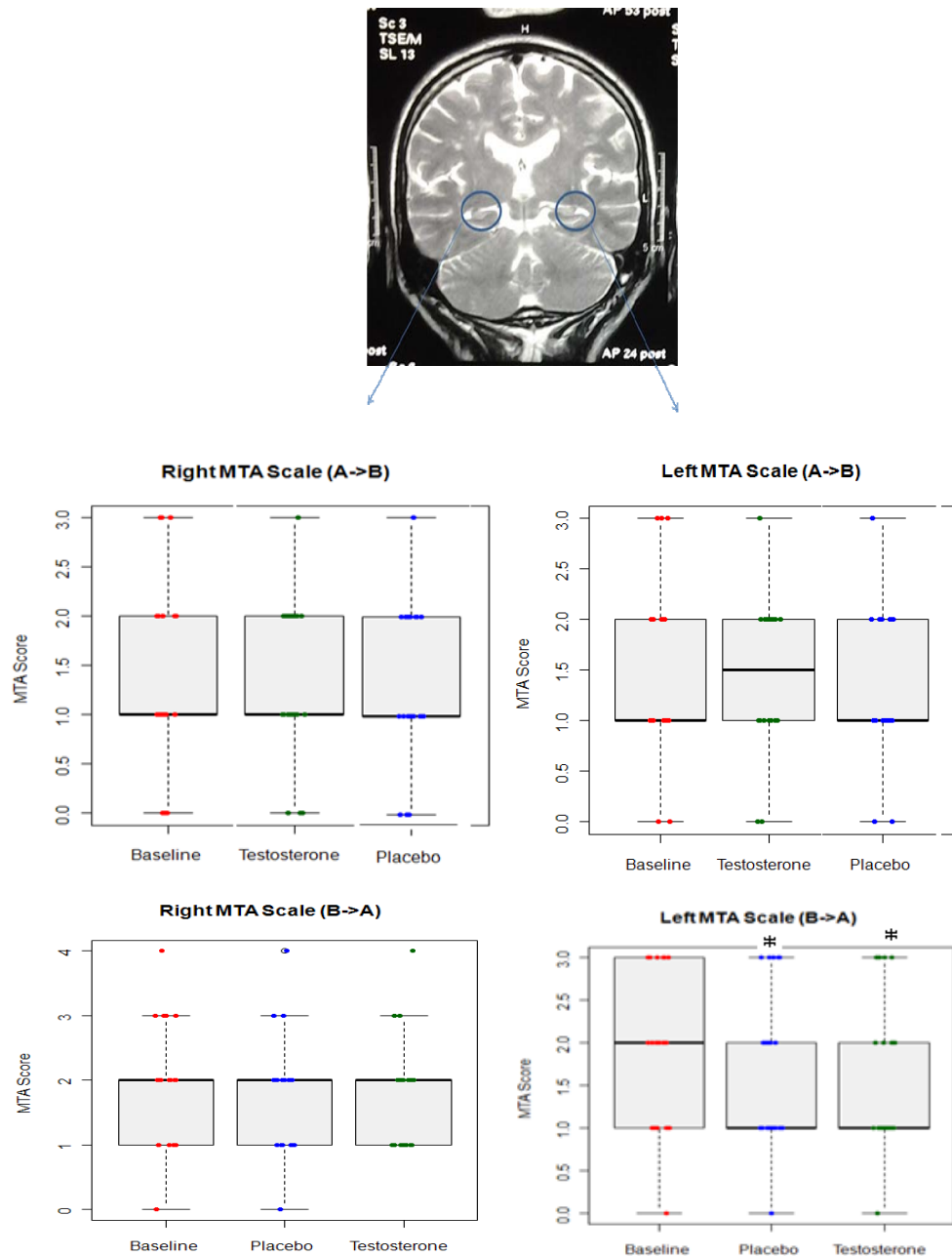


Figure 8.5: Boxplots of right MTA and left MTA scaling results (n=22) of subject brains from both arms of the study: treatment A→B (1st row) and treatment B→A (2nd row).

Significant reductions in atrophy were observed on the left MTA scale of treatment B→A, compared to baseline. There were no other significant differences observed. Data are presented in the scale range from 0-4 as described in Fig. 8.1, as medians \pm quartiles.

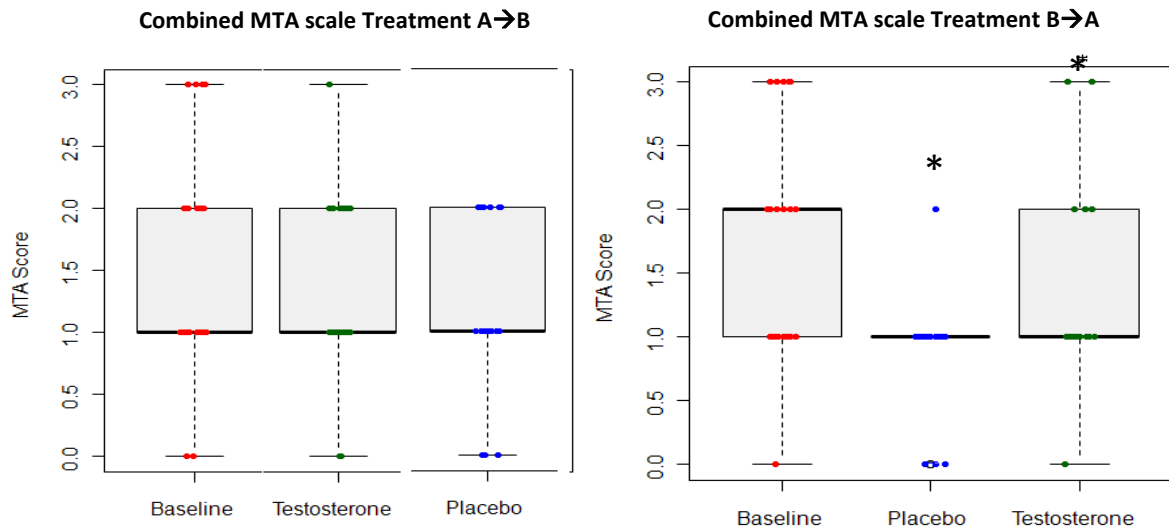


Figure 8.6 : Boxplots of combined right and left MTA scaling results (n=22) of subjects from both arms of the study: treatment A→B (left) and treatment B→A (right). Significant reductions in atrophy following testosterone and placebo treatments were observed only in arm treatment B→A compared to baseline; *p<0.05, in comparison to baseline.

Due to the small number of participants in the two arms of the study, the added statistical power that might be obtained following pooling of the MTA results of all participants from both arms of the study was also investigated.

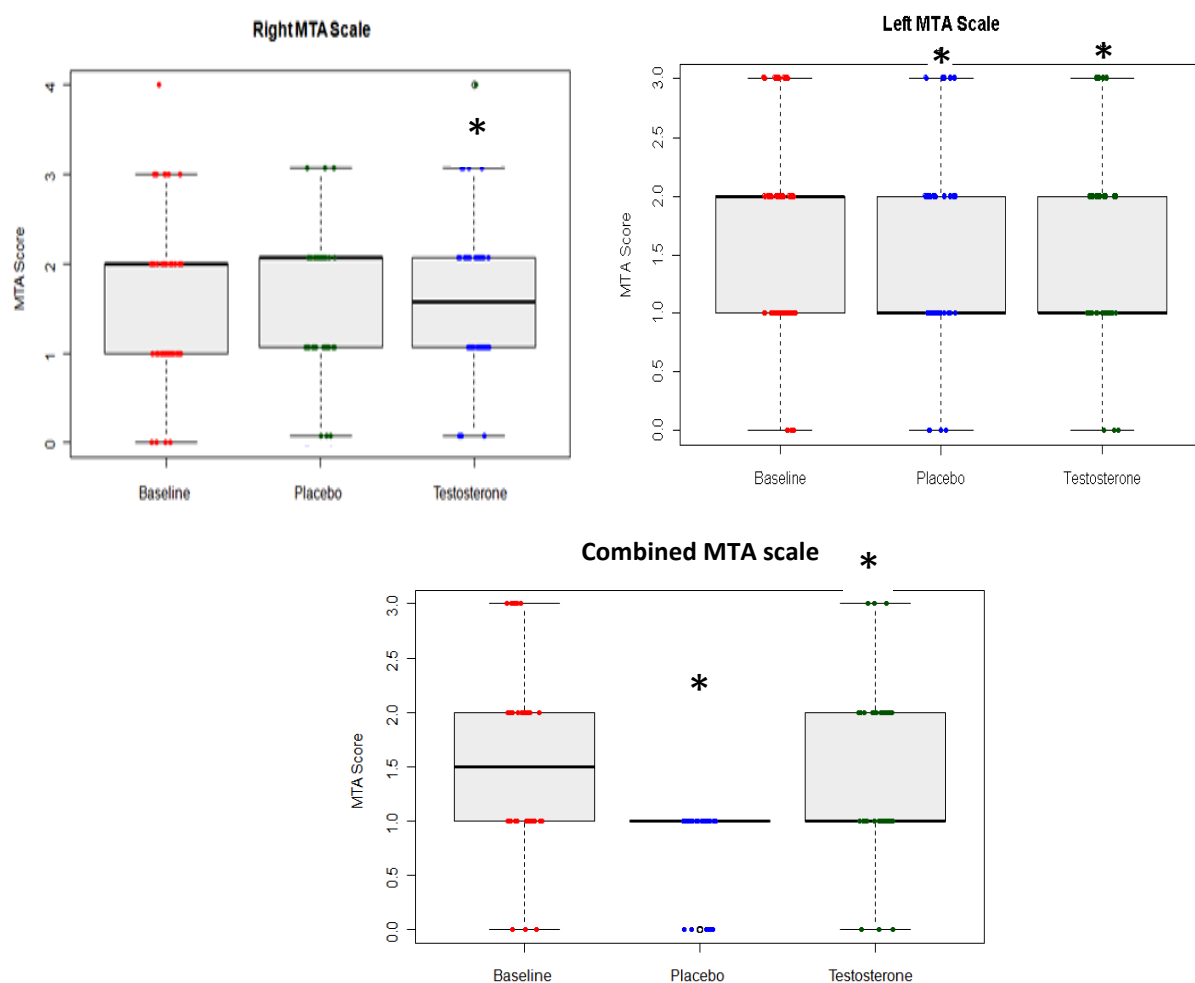


Figure 8.7: Boxplot of the combined MTA measurements (n=44) of both arms of the study: right MTA and left MTA (both on top row), and combined right and left MTA (2nd row). Data are presented using the scale range of 0-4 (as described in Fig. 8.1), showing the medians \pm quartiles. Significant differences were observed in the left MTA measurements, as well as the combined right and left MTA results following both treatments (testosterone and placebo) compared to baseline. In the right MTA scaling results, a significant reduction in MTA scaling was observed following testosterone treatment only. However, pooling the results from the two treatment arms no longer takes into account the order of the testosterone and placebo treatments; * $p < 0.05$ in comparison to baseline.

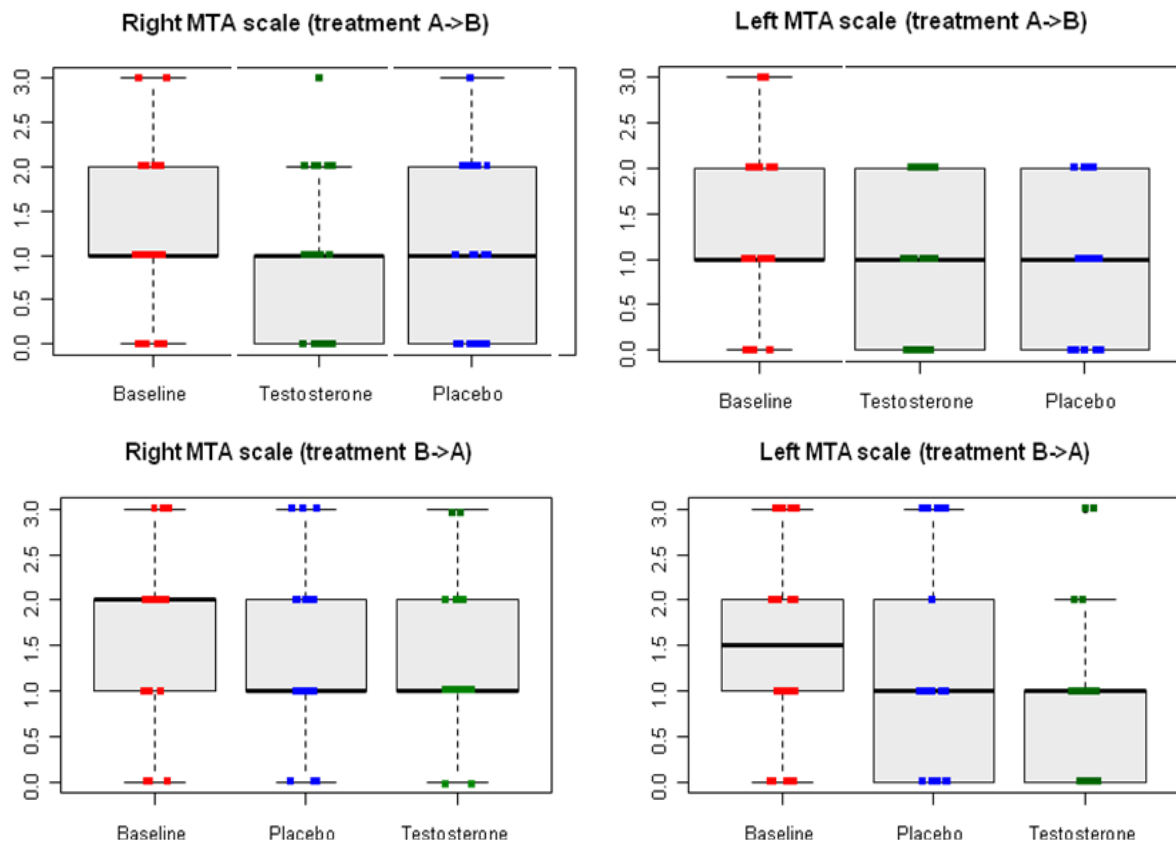
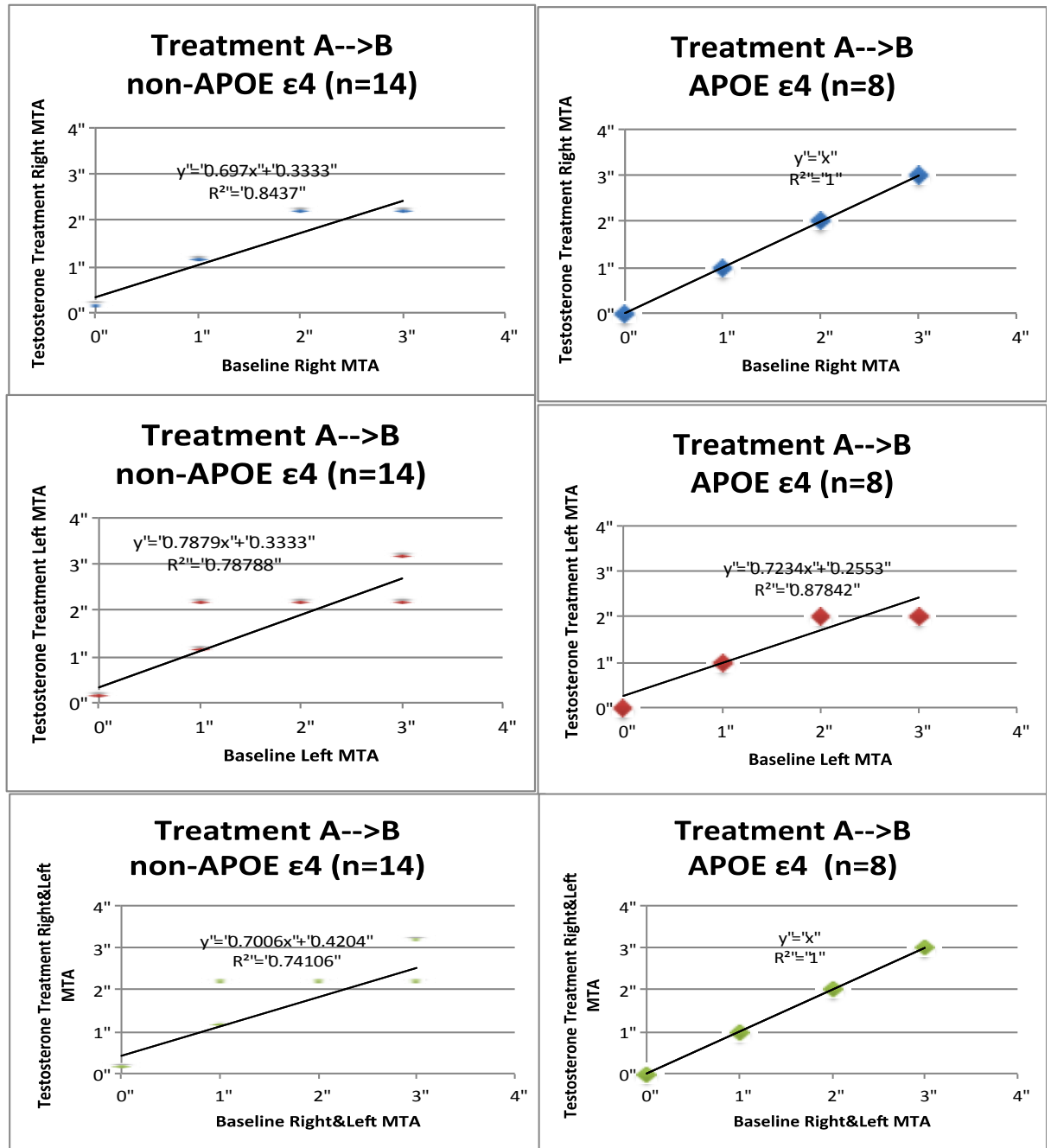


Figure 8.8: Boxplot of Right and Left Medial Temporal Atrophy (MTA) results for treatment A→B (1st row) and treatment B→A (2nd row), as assessed by rater 1. Data are presented in the scale range from 0-3 showing the median \pm quartiles.

The results of the Friedman Test for treatment A→B as well as treatment B→A indicated that there were no significant differences across the three time points (baseline, testosterone treatment, and placebo treatment) for the right MTA results: $X^2(2, n=22) = 1.5, p>0.005$, left MTA results : $X^2(2, n=22) = 4, p>0.867005$, and combined MTA results : $X^2(2, n=22) = 3.44, p>0.005$. However, there is a general trend suggesting that decreases in MTA scale results (i.e. reductions in MTA) could be achieved through testosterone treatment. This trend can be found in both arms of the experiment [treatment (A→B) and treatment (B→A)].

8.4.3 Baseline Medial Temporal Atrophy (MTA) scale as a predictor of testosterone treatment effect on subsequent rate of atrophy

(A)



(B)

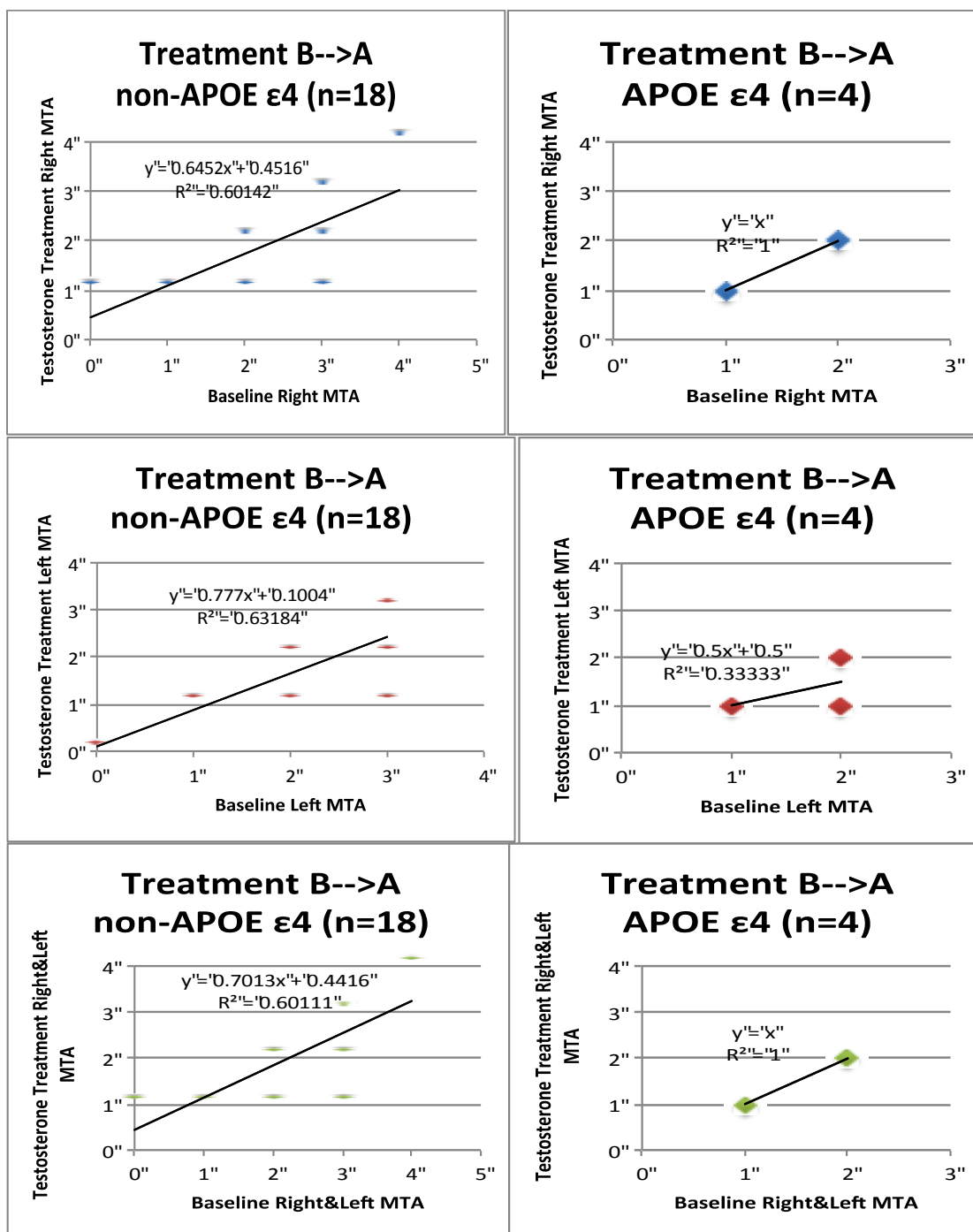


Figure 8.9: Scatter plots of linear regression analyses showing the levels of correlation between baseline MTA scale and testosterone treated-MTA scale results. The results have been grouped according to APOE ε4 status for both treatment A→B (A) and treatment B→A (B).

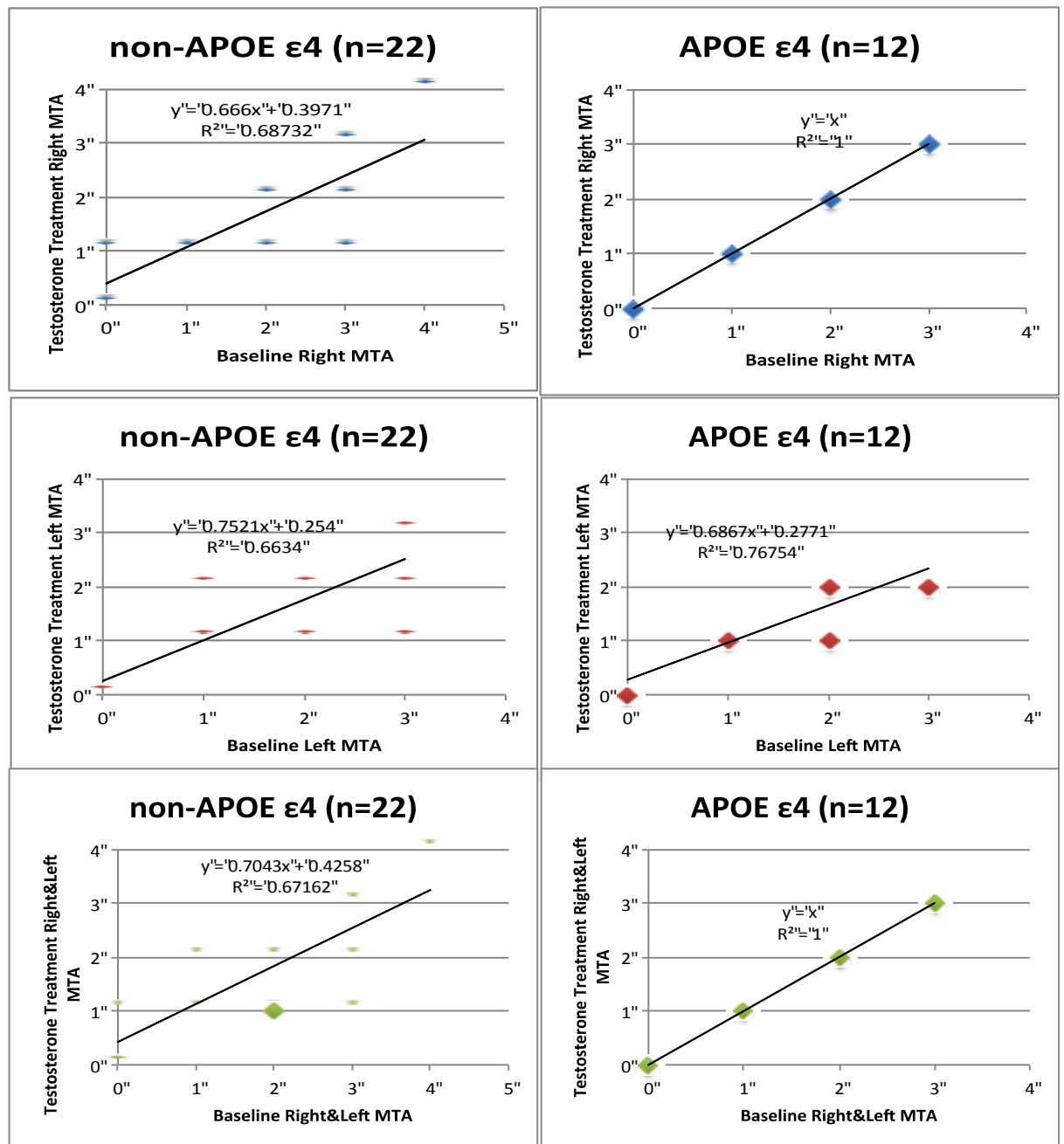


Figure 8.10 : Scatter plots of linear regression analyses showing the levels of correlation between baseline MTA scale and testosterone treated-MTA scale results, after pooling treatment A→B (A) and treatment B→A results. Participants are still grouped according to APOE ε4 status.

From Figure 8.9 and Figure 8.10 above, it appears that the non-APOE ε4 participants had a reduced amount of MTA in response to testosterone treatment compared to APOE ε4 participants.

8.4.4 Correlation between medial temporal atrophy and neuropsychological changes.

The cognitive function assessments of the subjects in this clinical trial were carried out at the same three stages as the MRI/MRS assessments. Thus cognitive function was assessed before any treatment had begun (baseline), then 24 weeks later after the first treatment of either testosterone (A→B group) or placebo (B→A group), then 52 weeks later after the second placebo (A→B group) or testosterone (B→A group) treatment. The neuropsychological tests carried out were the Mini Mental State exam (MMSE), Geriatric Depression Scale (GDS), and the Rey Auditory Verbal Learning Test (RAVLT). The results are listed below in Tables 8.1 to 8.9.

Table 8.1 Correlations of Right MTA scale with MMSE and GDS for Treatment A→B arm (n=22)

			RIGHT MTA Baseline A→B	RIGHT MTA End of Testo A→B	RIGHT MTA End of Placebo A→B
Spearman's rho	MMSE_BL_AB	Correlation Coefficient	-.053	.006	-.032
		Sig. (2-tailed)	.816	.978	.888
	MMSE_6_AB	Correlation Coefficient	-.301	-.293	-.320
		Sig. (2-tailed)	.173	.186	.147
	MMSE_9_AB	Correlation Coefficient	-.196	-.102	-.211
		Sig. (2-tailed)	.382	.653	.345
	GDS_BL_AB	Correlation Coefficient	-.166	-.148	-.168
		Sig. (2-tailed)	.461	.510	.454
	GDS_6_AB	Correlation Coefficient	-.139	-.160	-.117
		Sig. (2-tailed)	.538	.477	.603
	GDS_13_AB	Correlation Coefficient	.293	.250	.294
		Sig. (2-tailed)	.185	.262	.185
N (for all of above)		22	22	22	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8.2 Correlations of Left MTA scale with MMSE and GDS Treatment A→B (n=22)

			LEFT MTA Baseline A→B	LEFT MTA End of Testo A→B	LEFT MTA End of Placebo A→B
Spearman's rho	MMSE_BL_AB	Correlation Coefficient	-.072	-.084	.052
		Sig. (2-tailed)	.749	.711	.817
	MMSE_6_AB	Correlation Coefficient	-.392	-.385	-.265
		Sig. (2-tailed)	.071	.077	.234
	MMSE_9_AB	Correlation Coefficient	-.150	-.089	.008
		Sig. (2-tailed)	.504	.694	.973
	GDS_BL_AB	Correlation Coefficient	-.267	-.341	-.199
		Sig. (2-tailed)	.230	.121	.374
	GDS_6_AB	Correlation Coefficient	-.264	-.386	-.286
		Sig. (2-tailed)	.235	.076	.198
	GDS_13_AB	Correlation Coefficient	.280	.129	.288
		Sig. (2-tailed)	.207	.568	.193
N (for all of above)		22	22	22	

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table 8.3 Correlations of Combined MTA Scale with MMSE and GDS Treatment A→B (n=22)

			RIGHT&LEFT MTA Baseline A→B	RIGHT&LEFT MTA End of Testo A→B	RIGHT&LEFT MTA End of Placebo A→B
Spearman's rho	MMSE_BL_AB	Correlation Coefficient	-.076	-.044	.051
		Sig. (2-tailed)	.737	.844	.821
	MMSE_6_AB	Correlation Coefficient	-.359	-.329	-.243
		Sig. (2-tailed)	.101	.135	.276
	MMSE_9_AB	Correlation Coefficient	-.198	-.134	-.100
		Sig. (2-tailed)	.376	.552	.657
	GDS_BL_AB	Correlation Coefficient	-.267	-.214	-.122
		Sig. (2-tailed)	.230	.339	.589
	GDS_6_AB	Correlation Coefficient	-.234	-.226	-.142
		Sig. (2-tailed)	.295	.313	.530
	GDS_13_AB	Correlation Coefficient	.282	.192	.373
		Sig. (2-tailed)	.204	.392	.087
N (for all of above)		22	22	22	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8.4 Correlations Right MTA scale with MMSE and GDS Treatment B→A (n=22)

			RIGHT MTA BAaseline B→A	RIGHT MTA End of Placebo B→A	RIGHT MTA End of Testo B→A
Spearman's rho	MMSE_BL_BA	Correlation Coefficient	.065	.098	.016
		Sig. (2-tailed)	.773	.665	.945
	MMSE_6_BA	Correlation Coefficient	.054	.055	.048
		Sig. (2-tailed)	.810	.810	.833
	MMSE_13_BA	Correlation Coefficient	-.157	-.043	-.048
		Sig. (2-tailed)	.486	.850	.831
	GDS_BL_BA	Correlation Coefficient	-.319	.466 *	.508 *
		Sig. (2-tailed)	.148	.029	.016
	GDS_6_BA	Correlation Coefficient	.155	-.348	.328
		Sig. (2-tailed)	.491	.112	.136
	GDS_13_BA	Correlation Coefficient	.403	.444 *	.469 *
		Sig. (2-tailed)	.063	.038	.028
		N (for all of above)	22	22	22

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8.5 Correlations of Left MTA scale with MMSE and GDS Treatment B→A (n=22)

			LEFT MTA Baseline B→A	LEFT MTA End of Placebo B→A	LEFT MTA End of Testo B→A
Spearman's rho	MMSE_BL_BA	Correlation Coefficient	-.015	.004	-.070
		Sig. (2-tailed)	.949	.985	.756
	MMSE_6_BA	Correlation Coefficient	.192	.112	-.016
		Sig. (2-tailed)	.391	.621	.943
	MMSE_13_BA	Correlation Coefficient	-.048	.037	.049
		Sig. (2-tailed)	.830	.870	.830
	GDS_BL_BA	Correlation Coefficient	-.294	.303	.305
		Sig. (2-tailed)	.184	.170	.167
	GDS_6_BA	Correlation Coefficient	.212	.078	.037
		Sig. (2-tailed)	.344	.731	.869
	GDS_13_BA	Correlation Coefficient	.499 [*]	.455 [*]	.443[*]
		Sig. (2-tailed)	.018	.033	.039
		N (for all of above	22	22	22

****.** Correlation is significant at the 0.01 level (2-tailed).

*****. Correlation is significant at the 0.05 level (2-tailed).

Table 8.6 Correlations of Combined MTA scale with MMSE and GDS Treatment B→A (n=22)

			RIGHT&LEFT MTA Baseline B→A	RIGHT&LEFT MTA End of Placebo B→A	RIGHT&LEFT MTA End of Testo B→A
Spearman's rho	MMSE_BL_BA	Correlation Coefficient	.026	-.198	.075
		Sig. (2-tailed)	.907	.378	.740
	MMSE_6_BA	Correlation Coefficient	.201	-.392	.120
		Sig. (2-tailed)	.369	.072	.595
	MMSE_13_BA	Correlation Coefficient	-.166	-.204	-.058
		Sig. (2-tailed)	.459	.364	.797
	GDS_BL_BA	Correlation Coefficient	.334	.346	.293
		Sig. (2-tailed)	.129	.115	.185
	GDS_6_BA	Correlation Coefficient	.253	.224	.122
		Sig. (2-tailed)	.255	.316	.590
	GDS_13_BA	Correlation Coefficient	.449 *	.464 *	.386
		Sig. (2-tailed)	.036	.030	.076
		N (for all of above)	22	22	22

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table 8.7 Correlations of Right MTA scales with MMSE and GDS (n=44)

			RIGHT MTA	RIGHT MTA	RIGHT MTA
			Baseline	Testo	Placebo
Spearman's rho	MMSE_BL	Correlation Coefficient	-.020	-.010	.015
		Sig. (2-tailed)	.896	.948	.923
	MMSE_Testo	Correlation Coefficient	-.226	-.171	-.172
		Sig. (2-tailed)	.140	.267	.265
	MMSE_Placebo	Correlation Coefficient	-.146	-.075	-.126
		Sig. (2-tailed)	.345	.627	.416
	GDS_BL	Correlation Coefficient	.056	.161	-.147
		Sig. (2-tailed)	.717	.296	.342
	GDS_Testo	Correlation Coefficient	.145	.356 *	.164
		Sig. (2-tailed)	.349	.018	.288
	GDS_Placebo	Correlation Coefficient	.260	.311 *	.178
		Sig. (2-tailed)	.089	.040	.249
N (for all of above)		44	44	44	

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table 8.8 Correlations of Left MTA scale with MMSE and GDS (n=44)

			LEFT MTA Baseline	LEFT MTA Testo	LEFT MTA Placebo
Spearman's rho	MMSE_BL	Correlation Coefficient	-.060	-.085	.016
		Sig. (2-tailed)	.699	.584	.919
	MMSE_Testo	Correlation Coefficient	-.215	-.147	-.096
		Sig. (2-tailed)	.161	.342	.534
	MMSE_Placebo	Correlation Coefficient	-.035	-.072	.009
		Sig. (2-tailed)	.824	.644	.952
	GDS_BL	Correlation Coefficient	.011	.004	.073
		Sig. (2-tailed)	.943	.979	.640
	GDS_Testo	Correlation Coefficient	.134	.073	.115
		Sig. (2-tailed)	.385	.638	.456
	GDS_Placebo	Correlation Coefficient	.303 [*]	.069	.206
		Sig. (2-tailed)	.045	.658	.179
		N (for all of above)	44	44	44

^{**}. Correlation is significant at the 0.01 level (2-tailed).

^{*}. Correlation is significant at the 0.05 level (2-tailed).

Table 8.9 Correlations of Combined MTA scale with MMSE and GDS (n=44)

			RIGHT&LEFT MTA Baseline	RIGHT&LEFT MTA Testo	RIGHT&LEFT MTA Placebo
Spearman's rho	MMSE_BL	Correlation Coefficient	-.037	.004	-.019
		Sig. (2-tailed)	.811	.977	.901
	MMSE_Testo	Correlation Coefficient	-.258	-.184	-.191
		Sig. (2-tailed)	.091	.232	.214
	MMSE_Placebo	Correlation Coefficient	-.038	-.028	-.162
		Sig. (2-tailed)	.808	.856	.293
	GDS_BL	Correlation Coefficient	.035	.053	.083
		Sig. (2-tailed)	.824	.731	.592
	GDS_Testo	Correlation Coefficient	.130	.113	.136
		Sig. (2-tailed)	.401	.464	.378
	GDS_Placebo	Correlation Coefficient	.292	.141	.214
		Sig. (2-tailed)	.054	.361	.164
N (for all of above)		44	44	44	

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

From all the results above, it can be seen that in the treatment group B→A, there were positive correlations between subject's GDS and right and left MTA scale results, following testosterone treatment (Tables 8.4 and 8.5). In the treatment group A→B, this association was not seen. If the results from the patients in both arms are pooled according to treatment type (i.e., either testosterone treatment, or placebo treatment, regardless of treatment order), we found positive correlations between GDS results and right MTA scale results (but not left MTA scale results) following testosterone treatment. The number of subjects in this trial is small, but the positive correlations suggest that serial measurements of atrophy in the medial temporal lobe/hippocampus region will help to indicate whether testosterone reduces the rate of brain atrophy. Changes in brain atrophy appear to be reflected by changes in depression levels, as determined by the GDS. However, there were no significant correlations found between the MTA scale and either the MMSE or the RAVLT. While the finding reported in this chapter hold promise for the role of testosterone in protecting against atrophy of brain regions most susceptible to AD related neurodegeneration a larger study with a more robust neuropsychological battery will need to be undertaken for the clinical significance of this potential therapeutic approach to be determined.

8.5 Discussion

In this chapter, we have summarised results of a serial MRI study and a multiple voxel serial MRS study that were carried out on the low-testosterone subjects in this clinical study, in order to evaluate the effect of testosterone treatment on brain region atrophy and brain metabolite levels, compared to the original baseline levels. To our knowledge, this longitudinal study is the first that has involved the systematic acquisition of multiple serial spectra from patients in a testosterone clinical study. Brain metabolite levels (N-acetyl aspartate (NAA), choline (Cho) and creatinine (Cr)) were measured and the NAA/Cr and Cho/Cr ratios were calculated, to assess whether testosterone treatment could influence these parameters.

At baseline, lower NAA/Cr ratios in the left and right hippocampi and medial temporal lobe were observed, when compared with the thalamus white matter which provided “normal” spectra (control spectra - no pathologies observed). However, no significant alterations in Cho/Cr ratios were observed. These findings are consistent with a previous longitudinal study undertaken over a 13 month period¹⁴ which showed a greater decline in the NAA/Cr ratio in AD patients compared to controls (-1.8%/year in AD, 0.5%/year in controls) though non-significant rates of change were observed for Cho/Cr ratios in these same cases (1.2% in AD, 2.6% in controls). The reduction in NAA levels likely reflects neuronal loss or dysfunction. The 44 low-testosterone participants in this study did not have AD, but it needs to be remembered that they were subjective memory complainers, with MMSE scores ranging from 24-30, and a group average of 2. The 14 participants with MMSE scores <26 could probably be rated as approaching mild cognitive impairment. Decreased NAA levels have also previously been associated with increasing age in healthy participants.

Serially-acquired MRS, used as an outcome measure in this testosterone clinical trial, is dependent on a number of factors, including the variability and reproducibility of the technique. An ideal biomarker of neuroimaging should make it possible to, (1) detect early neurodegenerative pathology, (2) reflect pathological stage across the entire severity spectrum, (3) predict when an individual with early pathology may become demented, and (4) monitor the effect of a therapeutic intervention. Another property

relevant to an AD biomarker is to be able to differentiate between AD and other forms of dementia.

Studies of different common dementias have shown that NAA/Cr ratios are decreased in dementias that are characterized by neuron loss such as AD, frontotemporal lobe dementia (FTLD), and vascular dementia. It also seems that MI/Cr ratios are elevated in dementias that are pathologically characterized by gliosis such as AD and FTLD, whereas Cho/Cr ratios are elevated in dementias characterized by significant cholinergic deficit such as AD and dementia with Lewy bodies (Kantarci *et al.*, 2004).

Positron emission tomography imaging using PiB to bind to amyloid is currently more sensitive and recent studies suggest this is an excellent method to detect pre-clinical neurodegeneration. AD brain atrophy as detected by MRI both precedes and parallels cognitive decline. Significant plaque deposition also occurs prior to clinical decline. However cognitive decline is not directly due to amyloid deposition, rather the neurodegeneration that also occurs as part of the pathology of AD. Thus, PiB PET imaging and MRI could have complimentary roles in the pre-clinical diagnosis of AD (Jack *et al.*, 2009).

To date the potential ^1H MRS biomarkers have provided little or no advantages over other neuroimaging biomarkers. Thus, MRS biomarkers appear to be sensitive, however complimentary diagnostic tools are needed to help with diagnosis, as well as differential dementia diagnosis. Instead, recent studies in AD suggest that NAA may be more useful as a prognostic marker for monitoring neurodegeneration, stabilization, or improvement, and for evaluating therapeutic responses to novel drugs (Chen *et al.*, 2000).

Few longitudinal studies have used brain magnetic resonance spectroscopy as a predictor of conversion to dementia. One recent longitudinal study gave encouraging evidence that MRS (looking at the posteromedial bilateral parietal lobe and left medial occipital lobe) in MCI may identify patients at risk of early conversion to dementia (Modrego *et al.*, 2011). This study used MRS data at baseline and then followed-up 2 years later to examine rates of conversion from MCI to AD. Their results showed that the mean NAA/Cr ratio in the posteromedial bilateral parietal cortex was 1.38 in converters versus 1.49 in non-converters ($p < 0.0001$). From the results in this chapter,

the effects of successful testosterone treatment on brain metabolite levels are not yet clear. If, however, future studies demonstrate that NAA depletion or Cho elevation are reversed to a significant extent as a result of testosterone treatment, the measurement of these AD “biomarkers” using current MRS methodology may prove extremely useful in assessing treatment efficacy.

Unfortunately, MRS results of NAA/Cr ratio as well as Cho/Cr ratio did not show any significant changes following treatments in these current experiments. This may be due to the technical limitation of the low gradient 1.5 T MRI which was inadequate to exhibit the spectrum changes of brain metabolites. In order to acquire and detect brain metabolite changes accurately in future experiments, an MRI 1.5 T with a high gradient, or more preferably an MRI 3T may be necessary.

A study by DeCarli *et al.* (2007) has shown that qualitative ratings of MTA may have added impact in a clinical trial in a prospective and blinded manner for subjects with memory complaints, who may or may not be included in the MCI category. The MTA scale used in this study was simple, had good reliability, and could be easily implemented into standard clinical practice, albeit there were no alterations of MTA scale over time. Moreover, Schelten’s MTA scale has been compared to a computerized hippocampal volume measurement method and gave similar prediction accuracy (Westman *et al.*, 2011).

Animal studies have shown that testosterone treatment is indeed neuroprotective following motor neuron loss, and testosterone has been shown to attenuate somal and dendritic atrophy (Little *et al.*, 2009; Fargo *et al.*, 2007). However, there is still a lack of clinical studies which have investigated the effect of testosterone treatment on rates of brain atrophy. Based on published literature, this is the first study which has utilised the MTA scale to assess the effects of testosterone treatment on preserving brain structure. The MTA scale measurement showed no significant changes for either the right MTA, left MTA, or combined MTA of treatment A→B (Figs 8.5 and 8.6). With follow-up measurements at 6 month intervals or less as in this study, it is unlikely that small changes in brain anatomy would be detectable, even in the early stage of AD (Mizuno *et al.*, 2000). However, significant reductions in atrophy were observed in the left MTA and combined MTA of treatment B→A (Figures 8.5 and 8.6). as well as if we pooled

the participants of both arms (Figure 8.7). These results are unlikely to be due to the effect of testosterone treatment since the initial reduction in MTA was observed following placebo treatment (which in the B→A arm of the study was prior to testosterone treatment). The MTA changes that were observed over 6 months in this experiment are in accordance with results of a study by Murphy *et al.* 2010, who showed that increased MTA after six months was associated with subsequent memory decline in healthy elderly people. However, it is still unclear which region of the medial temporal lobe degenerates first in the earliest stage of AD.

The effect of APOE genotype was also investigated in this study since studies by Raz *et al.* 2005 and Du *et al.* 2005 have shown that both APOE genotype and age alter hippocampal atrophy and its rate of volume loss. Moreover, possession of APOE ε4 alleles has been shown to be associated with less frontal and more medial temporal lobe atrophy in AD (Geroldi *et al.*, 1999). In another study, Lehtovirta *et al.*, 1995 demonstrated that compared to healthy participants, ε4 homozygous people had more pronounced right hippocampal atrophy than ε4 heterozygous and non-APOE ε4 carriers. Such a gene dose effect of APOE ε4 has not been firmly established in non-demented elderly people. In this study, there were no differences observed in MTA scale results between APOE ε4 and non-APOE ε4 carriers. However, this is most likely due to the small number of participants in this study, resulting in a lack of statistical power to demonstrate significant differences between the APOE ε4 and non-APOE ε4 carriers. Nevertheless, these results were in agreement with a study by Lemaitre *et al.*, 2005 which found no effect of APOE ε4 on hippocampal atrophy in healthy elderly individuals. However, to date, associations between APOE genotype and medial temporal lobe atrophy rates (including the hippocampus) remain inconclusive.

The baseline MTA scale results were essential in determining the efficacy of testosterone treatment. The baseline MTA results could be interpreted as the sum of all structural changes occurring prior to the six months testosterone treatment. As shown in Fig 8.9 A&B, participants who started with high atrophy levels at their baseline measurement (MTA scale >2) showed a reduction in MTA scale following 6 months testosterone treatment in both arms, even after pooling all of the participants (Fig 8.10). This effect was observed in non-APOE ε4 carriers only but not in APOE ε4 carriers. These results may indicate that the carriers of the APOE ε4 allele may not be responsive

to testosterone treatment in terms of modifying medial temporal atrophy. In addition, longitudinal data by Cohen *et al.*, 2001 have shown that APOE ϵ 4 heterozygotes have greater annual rate of hippocampal volume loss compared to non-APOE ϵ 4 carriers. Therefore, another possible explanation for the results in this study in relation to APOE genotype is that testosterone may reduce the atrophy rate in APOE ϵ 4 carriers, but to a lesser extent than that observed in non-APOE ϵ 4 carriers.

For participants younger than 75 years, an MTA score of ≥ 2 is considered to be abnormal, whilst for participants greater than 75 years, a score of ≥ 3 is abnormal (Shonk *et al.*, 1995). The data distribution in this chapter showed that the cohort included men with normal as well as abnormal classifications in the age category of less than 75 years.

A psychological assessment of memory function and evaluations of structural changes in the medial temporal lobe are important for early diagnosis of AD. Therefore, the relationship between MTA and cognitive performance has been widely studied. In this experiment, no correlation between MTA and cognitive performance was found, as assessed by MMSE and RAVLT (see Tables 8.1-8). Concordantly, Crivello *et al.* 2010 did not find any interactions between APOE genotype or age and cognitive decline in the healthy elderly. A study by Mizuno *et al.* 2000 found no correlation between hippocampal volume and verbal memory. However, a recent study by Duchesne *et al.* 2009 did demonstrate a relationship between baseline MRI and MMSE after a 1 year follow-up using voxel-based morphometry (VBM).

In relation to depression, structural brain changes have been correlated with level of depression and cognitive performances. Lower depression scores (ie less depression) have been found to correlate with greater levels of medial temporal atrophy amongst those with cognitive impairment, suggesting that decreasing insight into cognitive status may lead to lower depression scores (Greig *et al.*, 2008). The results in the experiments here have shown a strong negative correlation ($p < 0.05$) between right and left MTA levels and depression levels as measured by the Geriatric Depression Scale (GDS); (although in treatment B \rightarrow A only, Tables 8.4 & 8.5). The results indicate that the lower the depression level, the lower the medial temporal atrophy was found to be following testosterone treatment. However, these results were only observed in treatment B \rightarrow A. It

is possible this could be due to the fact that the B→A group only had 4 APOE ε4 carriers (who as mentioned above have a greater likelihood of not responding as much to testosterone treatment), whereas the A→B group had 8 out of the 22 participants being APOE ε4 carriers.

The pattern of medial temporal atrophy observed in this study was shown to be improved to some extent by testosterone treatment. Future studies carried out on a larger cohort, possibly using longer treatment times and different doses, will determine the significance of the current results, and evaluate the full potential of testosterone treatment in the prevention or reduction of cognitive decline.

The results presented in this chapter have highlighted the importance of MRI as a powerful biomarker and MRS as a potentially useful biomarker for evaluating testosterone treatment. Both imaging biomarkers (MRI and MRS) would be more robust if combined with additional biomarkers (such as blood biomarkers or PiB-PET imaging). However, with MRS techniques being researched in earnest in recent years, longitudinal studies may soon provide earlier and more accurate MRS-based diagnostic tools. Future applications in the clinical setting may add such *in vivo* direct imaging techniques to allow more accurate assessment of hippocampus volume as well as brain metabolite changes, thus providing more useful diagnostic and treatment information.

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Chapter 9

General Discussion

Extensive research has been undertaken, particularly in the last 20 years, to improve our knowledge of androgen replacement therapy in the aging male, although conclusions from this research, on many aspects, remain controversial. A marked reduction in testosterone levels has a number of physiological and psychological effects. Recently, reduced testosterone has been linked to an increased risk of developing AD. This testosterone effect on AD risk is thought to arise from testosterone's numerous influences on the brain and on A β metabolism. Firstly, testosterone is produced in the testis, and a small amount reaches the brain and binds to the androgen receptor (AR) in target cells, and this has been shown to provide some neuroprotection (in some studies but not all) (Pike *et al.*, 2006). Testosterone may also be aromatized into estradiol (shown in many cell culture studies) (Haren *et al.*, 2002) and estradiol has been shown to influence A β levels (Gandy *et al.*, 2001). Importantly, testosterone levels are inversely correlated with plasma A β levels (Gillet *et al.*, 2003). Thus, many clinical trials and animal studies are currently being undertaken to assess the potential of testosterone as a neuroprotective agent.

In addition to testosterone, other hormones of the hypothalamic-pituitary-gonadal (HPG) axis such as luteinizing hormone (LH) have also gained attention as evidence has shown they may also influence the pathogenesis of AD. A marked reduction in testosterone levels causes disruption to the HPG axis. The subsequent loss of negative feedback by these hormones on the hypothalamus and pituitary leads to increased release of LH levels. Compared to age-matched controls, serum levels of LH have been shown to be higher in subjects with AD (Bowen *et al.*, 2000; Short *et al.*, 2001). Furthermore, increased serum LH levels have been associated with poorer cognitive performance in post-menopausal women (Rodrigues *et al.*, 2008) and serum LH levels have been found to correlate with plasma A β 40 levels in cognitively normal elderly men (Verdile *et al.*, 2008). Coupled with findings that gonadotropin releasing hormone (GnRH) agonists decrease A β levels in mouse brain (Bowen *et al.*, 2004; Casadesus *et al.*, 2006), these findings indicate that increased gonadotropin levels, particularly LH may be physiologically relevant in AD as they potentially modulate neurodegeneration in the aging brain. It is unlikely that any one of the hormones from the HPG axis plays a single and predominant role, but rather it may be a combination of hormones acting in concert.

While any specific underlying causes of AD are still yet to be determined, many risk factors have been identified. Three such risk factors, which are most likely inter-dependent, were investigated in this thesis: lack of testosterone, elevated LH levels due to the loss of negative feedback on the HPG axis, and increased A β levels. Many studies have examined the relationship between these three risk factors and the development of AD biomarkers. However, good randomized, double blind, placebo-controlled clinical studies in aging men are still lacking. The research presented in this thesis examined the cellular, structural and neuropsychological changes in response to exogenous testosterone supplementation using two models of testosterone depletion. The first involved *in vivo* validation of testosterone, LH and LH lowering agents in an animal model (castrated Guinea Pig). The second involved a trial of testosterone in testosterone deficient men with subjective memory complaints.

9.1 Effects of peripheral testosterone supplementation in castrated guinea pigs, which were used as an animal model for low testosterone levels and impaired HPG axis

A lack of the sex hormone testosterone results in increased pathology characteristics of AD, including decreased expression of APP in the brain (particularly the hippocampus), increased production of APP-CTF fragments and increased cerebral A β levels (Drummond *et al.*, 2009). The results presented in Chapter 3 show the effect of exogenous testosterone administration on testosterone levels, A β , and APP and its metabolites in castrated guinea pigs. As expected, castration of the guinea pigs (GDX) caused a significant reduction in plasma testosterone levels, and the administration of high levels of testosterone increased the plasma testosterone levels which progressively returned to the non-GDX level. CSF A β 40 levels in the guinea pigs increased after castration and levels correlated with low testosterone levels. The administration of testosterone, at either a low dose or high dose significantly reduced the CSF A β 40 levels to non-GDX levels after 36 days. Nevertheless, the A β 40 levels in plasma increased significantly following testosterone administration in both the low dose and

high dose testosterone supplementation. Thus, the testosterone appears to decrease A β 40 levels in the CNS, yet increase A β 40 levels in the plasma. This phenomenon is considered to be the result of rapid clearance from the brain, nevertheless further, more extensive studies are needed to confirm this observation. The results suggest strongly that the measurement of blood plasma A β levels only is not necessarily appropriate or sufficient for the monitoring of testosterone therapy for AD prevention.

Interestingly, A β levels were found to be high in GDX hippocampi after 18 days of low-level testosterone supplementation, yet A β levels did normalize after 36 days. In contrast, high-level testosterone supplementation resulted in a decrease in A β levels after 18 days, with the same lower levels measured again at 36 days of supplementation, suggesting A β levels had stabilised earlier on the higher dose. These findings suggest that high testosterone levels can regulate A β levels in the blood and brain, especially in the hippocampus, and that the testosterone levels can influence the time taken for normal A β levels to be reached. Testosterone supplementation in the GDX was also found to increase levels of α -APPs in the frontal cortex. This result suggests that testosterone supplementation can influence the metabolism of α -APPs, an APP cleavage product known to have a neuroprotective role, Increasing production of α -APPs has also been associated with a decrease in production of A β . The results from Chapter 3 provide further evidence of a role for reduced testosterone levels in AD pathogenesis.

9.2 Effects of CNS as well as peripheral administration of LH in castrated guinea pigs

There is growing evidence that increases in gonadotropin levels associated with aging may also contribute to AD pathogenesis. The results in Chapter 4 support this notion, as results demonstrated that direct exposure of the brain to LH can cause a significant increase in A β production, as measured in the frontal cortex and in the hippocampus, resulting in an increase in CSF and cerebral A β levels. Furthermore, APP metabolites were found to be altered in the hippocampus 28 days after LH administration. Hence, the hippocampus appears to be a major target for LH. Unfortunately, LH levels couldn't be directly measured in the guinea pigs. This study has revealed that LH administration can increase A β production in the brain, yet in this case there were no

increases in A β levels in the blood. Studies of LHR (-/-) APPsw mice have shown that removing the LH receptor results in lower A β plaque deposition, less astrogliosis and less tau phosphorylation (Lin *et al.*, 2010).

It is not clear how LHR ablation reduces A β levels. The mRNA levels of enzymes involved in the amyloidogenic and non-amyloidogenic APP cleavage pathways were measured in the LHR (-/-) mice, as well as mRNA levels of A β degrading enzymes such as insulin degrading enzyme and neutral endopeptidase. These mRNA levels were found to be unchanged, suggesting post-translational mechanisms are involved in the changes in A β levels. LH works not only through the LHR, but also through other pathways that may influence the amyloidogenic process, thus the LH signalling pathways in neurons are still not well understood. Other possibilities that need further investigation include the role of astrocytes and microglia in removing A β from the brain. Astrogliosis is believed by many to be a reaction to A β deposition, yet astrogliosis is also known to inhibit microglial phagocytosis, an effect also likely to reduce A β removal. Therefore, the mechanisms by which LH modulates A β metabolism remain to be determined.

9.3 Effects of a peripheral LH lowering agent in castrated guinea pigs

Chapter 4 provided further evidence that LH levels can influence AD pathogenesis, as direct application of human LH to the guinea pig CNS resulted in an accumulation of A β in a similar manner to that reported for neuronal cells in culture and *in vivo*. In Chapter 5 we did further investigations of LH, using LH implanted in the periphery. In contrast with results in Chapter 4, the application of human LH to the guinea pig periphery resulted in reduced cerebral A β levels. Although not consistent with findings obtained in mouse models, it provides an interesting line of research that can be further explored in the guinea pig.

Leuprolide, a potent GnRH agonist, was also used in this study to investigate further the role of LH in guinea pigs. *In vivo* studies of leuprolide, as a suppressor of LH (to lower endogenous LH (Ravivarapu, 2000), found that administration to female mice causes a decrease in cerebral A β levels (Bowen *et al.*, 2004; Casadesus *et al.*, 2006). The results

of the experiments showed that leuprolide treatment can reduce A β 40 levels in the brains of guinea pigs, however the effect appears to be limited to the frontal cortex. The concomitant trend toward increased α -APPs levels indicates that leuprolide may be promoting the non-amyloidogenic pathway.

Testosterone reduces cerebral A β levels (Chapter 3) and LH increases CSF and cerebral A β (Chapter 4). To explore these findings further, we tried to determine whether there are additional benefits when these two treatments are combined, in other words, we wished to determine if the effects are additive. Additional results from Chapter 5 suggest that there are no additional benefits with respect to A β levels when testosterone treatment is combined with leuprolide treatment. However, the combined treatment may be promoting the non-amyloidogenic pathway further, as indicated by a greater increase in α -APPs levels. Taken together, this animal study has added further evidence of the role of testosterone and LH on regulating A β . It has also shown a promising result for future clinical study by modulating the testosterone as well as LH levels.

9.4 Effects of testosterone supplementation on blood biomarkers in testosterone deficient men

There are lessons to be learned not only from the animal studies but also from clinical studies which have investigated the effects of testosterone replacement therapy in men. It is well known from the animal studies that the effects of testosterone mediated through androgen receptors (AR) are widespread and complex, and influence certain aspects of cognition and behaviour. Studies of exogenous testosterone administration in men have provided mixed results. However, from studies of people with Subjective Memory Complaints (SMC), Mild Cognitive Impairment (MCI) or AD, there does appear to be an association between low testosterone levels and (increased) risk of AD. A more comprehensive investigation of hormones, lipid profiles and related proteins, such as total testosterone, dihydrotestosterone (DHT), estradiol, LH, cholesterol, LDL, HDL, insulin, apolipoprotein E, sex-hormone binding globulin, and albumin is required

to obtain a more complete picture of the interactions between the components of the HPG axis, and to characterise better the effects of these interactions on A β metabolism.

From our clinical trial on elderly testosterone deficient men (Chapter 6), testosterone treatment for 6 months caused a significant increase in plasma total testosterone levels, which returned to baseline levels when the treatment was terminated. Similar results were observed with DHT, an active testosterone metabolite, also a potent androgen that cannot be aromatized to estrogen (Cunningham, 1989). However, a significant rise in estradiol levels was not seen, which suggests there must have been greater conversion of testosterone into DHT. These results are not unexpected, as the route of testosterone administration favours this outcome: transdermal testosterone administration (as opposed to intramuscular or by pellet) normally only raises estradiol levels slightly, while quadrupling DHT levels, due to 5 α -reductase enzymes in the skin (Swerdlow *et al.*, 2000). Consequently, testosterone treatment also resulted in a significant decrease in LH levels, through the feedback mechanism on the HPG axis.

Testosterone and DHT treatment has been shown to influence lipoprotein levels, early studies showed that both HDL and LDL levels could be lowered by transdermal DHT treatment for 3 months (Vermuelen *et al.*, 1985). Later studies then showed that the effect of testosterone on lipoprotein metabolism was highly dependent on whether the administration of testosterone resulted in physiological or supraphysiological concentrations (Alexandersen *et al.*, 2004). In accordance, the testosterone therapy carried out in this study showed significant effects on HDL, such that a significant decrease in levels was observed following testosterone treatment. However, out of the lipoprotein components measured in these experiments, only HDL was found to change significantly – there were no significant differences observed in cholesterol, triglyceride, or LDL levels. In addition, testosterone treatment did not alter the Chol/HDL or LDL/HDL ratios, recognized as major risk factors for cardiovascular disease. The effects of testosterone treatment were not limited to lipoprotein metabolism, insulin pathways were also affected. In accordance with previous studies which showed that testosterone therapy can improve insulin sensitivity (Shabsigh, 2005; Pasquali *et al.*, 1991; Simon *et al.*, 1992) the results from this study revealed a trend towards increased insulin sensitivity following testosterone treatment. However, it is important to note that the more one looks into relationships between testosterone

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replacement therapy and cholesterol, LDL, HDL, and insulin, the more complex and variable the effects seem to be. The effects can vary due to the methods of testosterone preparation and administration, the testosterone doses used, the duration of testosterone treatments, and the ethnicity and age of the participants (Robert *et al.*, 2000; Minneman *et al.*, 2008; Makinen *et al.*, 2007).

In the elderly male, prostate cancer is common and its incidence increases with increasing age (Bowen *et al.*, 2000). Therefore a potential problem in terms of testosterone treatment in elderly men is whether testosterone treatment increases the risk of developing prostate cancer. The effects of testosterone treatment on the prostate are very important when considering its safety, and for this purpose PSA (prostate specific antigen) levels should be carefully monitored. The results in this study suggest that PSA levels do not increase as a result of testosterone treatment, when carried out as done in this study. Therefore, these results together with the almost unchanged lipid profile suggest that the application of 100mg testosterone cream each day for 6 months is safe and does not have any adverse effects.

Testosterone treatment was not found to influence plasma A β levels significantly. Both A β 40 and A β 42 levels were measured and not found to be significantly different, although there seemed to be a trend towards decreasing levels following testosterone treatment.

This study hoped to estimate effects of testosterone treatment on CSF markers of AD. The CSF biomarkers or analytes to be tested included A β 42, total tau, phospho-tau, tau/A β 42, and phospho-tau/A β 42. Unfortunately CSF collection is not a comfortable procedure, and too few subjects were keen to have their CSF tested. The data that was obtained includes CSF data from three healthy (subjective memory complainer) participants from the main clinical study and two participants who have been diagnosed with AD from a side study (results not shown). As expected, AD participants had significantly lower baseline CSF A β 42 levels when compared to the healthy participants. These low CSF A β 42 levels reflect deposition of A β in plaques (Strozyk *et al.*, 2003; Tapiola *et al.*, 2009). Overall, the testosterone treatment showed a trend towards lowering CSF A β 42 levels (although there was variation between individuals), and raising the plasma A β 42 levels in both the AD and non-AD participants. The

number of participants clearly lowers the significance of these observations, yet the observations are nevertheless encouraging. High CSF total tau and phospho-tau levels would normally be expected in AD patients as a reflection of active axonal and neuronal damage (Blennow *et al.*, 1995). However, in this study, total tau and phospho-tau levels in the AD participants were almost the same as those of the healthy (subjective memory complainer) participants. In terms of testosterone effect on tau levels, this study demonstrated that testosterone lowers tau (but not phospho-tau levels) in the healthy participants, yet had no effect in the 2 AD participants (results not shown). Meanwhile, changes in tau/A β 42, and phospho-tau/A β 42 were not observed.

ApoE plays an important role in the periphery as a mediator of lipoprotein metabolism and lipid clearance. In the central nervous system, apoE plays a role in the redistribution of lipids and cholesterol during membrane repair and it is believed to be important for maintaining synaptic plasticity especially after neuronal injury. ApoE's importance in the central nervous system is underscored by the low abundance of other apolipoproteins. ApoE has been linked to AD in many ways. One of the first indications that apoE might be involved in AD came from the immunochemical localization of apoE in extracellular amyloid plaques and in neurons containing neurofibrillary tangles (Wisniewski, 1992). It was then discovered that possession of APOE4 alleles meant a higher risk of AD, and it was soon described as the main risk factor for late onset AD. Many studies then found that, compared to the more common apoE3 isoform, apoE4 enhances A β aggregation, increases A β deposition, and reduces A β clearance (Schemechel, 1993; Berr *et al.*, 1994), whereas ApoE2 has a protective effect (Corder *et al.*, 1993). ApoE4 is also associated with poor neuronal repair. The mechanism whereby apoE4 promotes AD is not yet established, it may involve more than one of the above factors. Considerable evidence supports all of the above mechanisms. For example, *in vitro* studies have also shown that all apoE isoforms inhibit A β aggregation with apoE4 being less effective than apoE3 (Ma *et al.*, 1994; Moir *et al.*, 1999; Sanan *et al.*, 1994). Thus, the exact role of apoE is yet to be elucidated.

APOE allele status was also taken into account in this study. There were no significant differences observed between APOE ϵ 4 carriers and non-APOE ϵ 4 carriers for all of the blood measurements as well as BMI and body fat percentage. Participants with APOE ϵ 4 had a slightly higher BMI and body fat percentage when compared to participants not carrying APOE ϵ 4. Interestingly, there was no significant increase of total testosterone levels in the APOE ϵ 4 participants when compared with those who were non-APOE ϵ 4. The results suggest that APOE ϵ 4 status might not be a determinant of testosterone therapy response in this study. However, this result may have occurred due to the small as well as unequal number of participants in regards to APOE ϵ 4 allele status. Furthermore, conflicting conclusions concerning the potential effects of APOE ϵ 4 allele status may also have arisen from differences in clinical study design.

9.5 Effects of testosterone supplementation on cognitive performances and quality of life in testosterone deficient men

It has been known since the 1970s that circulating testosterone levels decline gradually with age. More recently, the relationship between low levels of testosterone and cognitive decline has been the subject of intense research since a number of studies showed that hormone replacement therapy could reduce the risk of developing AD (in men as well as in women). The results from Chapter 7 have added to the evidence indicating that testosterone can improve cognition and quality of life in the elderly. Cognitive function of the study participants (in general) improved with testosterone therapy. Testosterone deficient men given testosterone treatment (in both arms of the study) showed a significant increase in their MMSE scores. Neither age nor education was a statistically significant factor when added as a covariate. Previous studies have produced similar results, as high testosterone has been found to be associated with improved performance in several cognitive tests (Barret-Connor *et al.*, 1999), when compared to men with lower plasma testosterone levels (Yaffe *et al.*, 2002). For example, testosterone supplementation in elderly men has been shown to improve performance in working memory tasks (Janowski *et al.*, 2000) and to improve spatial cognition (Janowski *et al.*, 1994). However, one study found that testosterone treatment

had no effect on verbal memory and was negatively associated with verbal fluency in men (Wolf *et al.*, 2000). In line with Wolf *et al.*, 2002, testosterone treatment in this study have not shown an improvement in verbal memory.

In terms of quality of life, testosterone treatment was found to reduce depression significantly in both arms of the study, as measured by Geriatric Depression Scale (GDS). These results are in accordance with another study in which androgen depletion by chemical castration resulted in an increase in depression (Almeida *et al.*, 2003). However, although testosterone treatment has been shown to improve muscle strength, bone mineral density and some other symptoms of hypogonadism, improvements in depression are not consistently found (Seidman, 2007) . In terms of physical health and mental health, testosterone does not seem to have a significant effect in this study.

Overall, general cognitive screening instruments, which include the MMSE, RAVLT, GDS, and SF36 have proven to be beneficial for monitoring effects of testosterone treatment on cognitive function when used in a patient population with low testosterone levels, who are at risk of cognitive impairment due to either age or the presence of memory dysfunction.

9.6 Effects of testosterone supplementation on AD-related brain metabolites and medial temporal structure in testosterone deficient men

Some of the more prominent early features of AD include mild memory impairment, structural atrophy of the medial temporal lobe and changes in the levels of certain brain metabolites such as myo-inositol (mI, thought to be a glial marker, increases in AD) and N-acetyl aspartate (NAA, decreases in AD, interpreted as a marker of neuronal loss) (Mizuno *et al.*, 2000). Magnetic resonance spectroscopy (MRS) is used to detect changes in NAA/creatine or mI/creatine, yet in our measurements summarised in Chapter 8, it can be seen that no significant changes were detected in brain metabolite levels following testosterone treatment. On the other hand, medial temporal atrophy (MTA) was assessed using a standardised visual rating scale, and it was found that testosterone treatment resulted in a reduction in atrophy by decreasing the MTA score

by 1, regardless of sequence effect of the treatment (Chapter 8). Medial temporal lobe atrophy seen in MRI is indeed macroscopic result of neuronal loss and synapses (Bobinski *et al.*, 2000)

The presence of APOE $\epsilon 4$ allele participants was investigated for the response to testosterone treatment. Participants who started with high atrophy rates at their baseline (MTA scale >2) have shown reduction of MTA scale following 6 months testosterone treatment in both arms, even after pooling all of the participants. This effect was observed in non-APOE $\epsilon 4$ carriers only but not in APOE $\epsilon 4$ carriers. These results may indicate that the carriers of the APOE $\epsilon 4$ allele may not be responsive to testosterone treatment in terms of modifying medial temporal atrophy. Another hypothesis is testosterone effect on APOE $\epsilon 4$ participants (which have greater rates of atrophy compared to non-APOE $\epsilon 4$) (Cohen *et al.*, 2001) may only maintain the integrity of medial temporal lobe structure, thereby preventing its atrophy rates.

In Chapter 6, a significant increase in the number of red blood cells was found to occur following testosterone treatment. This increase in red blood cells is believed to help regenerate stem cells proposed to stimulate hippocampal neurogenesis, which one might assume would be reflected in an improvement in the MTA scale. However, given the smaller potential of neurogenesis (DeCarolis, 2010), one can probably assume that the effect of testosterone treatment as measured here is due to a reduction in brain atrophy.

In relation to cognitive performance and quality of life, testosterone treatment was found to decrease depression to some extent, and in fact a positive correlation was found between the reduction in medial temporal atrophy and the decrease in depression. This supports previous studies which showed that stress or depression can damage the hippocampus, leading to its atrophy (McEwen, 1999).

This study has demonstrated that medial temporal structure changes can be detected even after a short time period. Such changes may allow assessment of a drug's effect in the prodromal phase of AD, such that any positive effects on atrophy may be measurable prior to the appearance of detectable cognitive changes. Taken together, this study has found that MRI, but not MRS may provide a potentially powerful biomarker for early stages of AD.

9.7 Conclusions

Our understanding of testosterone replacement therapy in the aging male has greatly improved over the last 20 years. However, good randomized, double-blind, placebo-controlled clinical studies are still lacking. In Indonesia in particular, no such testosterone clinical study as a potential approach for the prevention and treatment of AD has been carried out till now. There is a need for longitudinal clinical studies to help us to understand better whether testosterone replacement therapy is of value in the prevention, delay and/or treatment of AD. This thesis has added to the growing evidence that testosterone replacement in testosterone deficient patients (as well as in animal models) has potential benefits with respect to brain health and cognition, and has increased our understanding of likely side-effects. A combination of biomarkers : blood measurements, neuropsychological assessment, CSF A β 42 and tau measurements (although in very limited samples), and neuroimaging tools (MRI and MRS) investigated in this double-blind randomized placebo-controlled crossover study has shown these markers to be most powerful tools in the early diagnosis of AD, in the understanding of the pathophysiology of AD, and in the monitoring of AD treatment.

This study has also provided further evaluation of the safety of testosterone replacement therapy, with results suggesting the therapy utilised here causes no increased risk of prostate cancer or increased cardiovascular risk. Hence, if this study is initiated in the right “critical time” following testosterone deficient men with future suggestions applied, it will be a significant future implications for prevention as well as early detection of AD. Taken together, the animal studies and human studies in this thesis have demonstrated and explored further the links between testosterone, LH, and A β , three main players in the pathogenesis of AD. Furthermore, this study suggests that, with the aid of AD biomarkers mentioned above, there may be a window of opportunity for the pre-symptomatic detection of AD, and the prevention, and/or delay of AD symptoms in elderly testosterone deficient men using testosterone replacement therapy.

Other potential treatments that can enhance androgen status include non-steroidal, small molecule compounds (selective androgen receptor agonists (SARMS)). These have high affinity for the androgen receptors in the brain. There is the potential to signal through the androgen receptor and only activate essential pathways (e.g. anabolic pathways in the brain). SARMS are not commercially available yet, although investigations are under way into the therapeutic implications for androgen deficient males, including those with memory complaints. SARMS may also be safer for patients, since there is no potential prostate stimulation. 7 α -methyl-19-nor-testosterone (MENT), which is less likely to stimulate the prostate, is 10 times more potent than testosterone.

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APPENDICES

Table 1 Individual Testosterone levels Treatment A→B

Patient	APOE	T Baseline AB	Mean T Testo AB	Mean T Placebo AB	Treatment effect AB*
1	E4 -	9.53	24.48	8.10	16.38
2	E4 -	14.21	32.12	18.69	13.43
3	E4 -	25.97	37.67	23.09	14.58
4	E4 -	19.87	18.38	15.61	2.76
5	E4 -	17.23	16.95	14.32	2.63
6	E4 -	23.09	26.29	17.88	8.41
7	E4 -	17.44	23.02	19.91	3.11
8	E4 -	20.04	25.67	15.81	9.86
9	E4 -	17.72	30.31	18.34	11.97
10	E4 -	11.68	9.01	10.37	-1.35
11	E4 -	14.42	20.31	12.78	7.53
12	E4 -	16.33	28.83	14.89	13.94
13	E4 -	13.24	20.03	19.23	0.80
14	E4 -	9.85	21.56	9.78	11.78
15	E4 +	16.75	27.58	15.36	12.22
16	E4 +	22.09	38.73	21.54	17.18
17	E4 +	18.34	42.33	16.64	25.69
18	E4 +	21.67	31.76	20.19	11.57
19	E4 +	13.83	37.56	13.12	24.44
20	E4 +	16.09	39.05	15.56	23.50
21	E4 +	10.82	20.05	10.27	9.77
22	E4 +	12.07	30.43	10.09	20.34

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

Only one participant had lower testosterone levels compared to placebo (yellow box).

Table 2. Individual Testosterone levels Treatment B→A

Patient	APOE	T Baseline BA	Mean T Placebo BA	Mean T Testo BA	Treatment effect BA*
1	E4 -	17.13	17.70	67.84	50.14
2	E4 -	24.03	19.27	30.78	11.51
3	E4 -	11.55	11.27	30.26	18.98
4	E4 -	14.98	13.02	18.09	5.07
5	E4 -	24.89	21.21	31.90	10.69
6	E4 -	10.30	13.33	16.25	2.92
7	E4 -	14.73	15.36	15.64	0.27
8	E4 -	21.81	28.20	27.97	-0.23
9	E4 -	19.10	17.66	22.47	4.80
10	E4 -	22.78	24.55	47.95	23.40
11	E4 -	14.46	13.46	26.06	12.60
12	E4 -	20.94	18.10	33.82	15.72
13	E4 -	19.31	19.00	23.28	4.28
14	E4 -	27.77	28.52	22.70	-5.82
15	E4 -	23.85	20.22	48.47	28.24
16	E4 -	10.40	12.67	16.58	3.92
17	E4 -	17.96	17.55	33.30	15.74
18	E4 -	11.06	14.32	17.95	3.62
19	E4 +	25.34	21.98	29.48	7.51
20	E4 +	9.08	15.53	23.11	7.59
21	E4 +	14.60	15.08	9.60	-5.47
22	E4 +	21.29	16.07	8.78	-7.29

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

Four participants had lower testosterone levels compared to placebo (yellow boxes).

Table 3. Individual DHT levels Treatment A→B

Patient	APOE	DHT Baseline AB	Mean DHT Testo AB	Mean DHT Placebo AB	Treatment effect AB*
1	E4 -	0.90	7.06	1.17	5.89
2	E4 -	2.53	11.75	2.59	9.16
3	E4 -	3.57	11.93	2.38	9.55
4	E4 -	2.43	6.58	2.02	4.55
5	E4 -	2.36	4.30	1.35	2.95
6	E4 -	1.04	9.03	1.29	7.74
7	E4 -	1.59	8.71	2.87	5.84
8	E4 -	2.57	8.96	1.40	7.56
9	E4 -	3.05	8.77	1.81	6.96
10	E4 -	1.42	2.40	1.76	0.64
11	E4 -	1.59	10.01	1.62	8.39
12	E4 -	1.59	7.87	2.07	5.80
13	E4 -	0.60	5.27	1.71	3.56
14	E4 -	0.62	5.48	1.55	3.92
15	E4 +	3.22	16.77	2.32	14.45
16	E4 +	2.08	16.81	1.58	15.23
17	E4 +	3.16	15.19	2.92	12.26
18	E4 +	1.77	10.86	1.51	9.34
19	E4 +	1.53	11.82	0.26	11.56
20	E4 +	1.18	6.78	1.14	5.63
21	E4 +	0.67	5.15	1.05	4.10
22	E4 +	1.03	8.40	1.02	7.37

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

All participants had some increase in DHT levels, although the increase did vary widely.

Table 4. Individual DHT levels Treatment B→A

Patient	APOE	DHT Baseline BA	Mean DHT Placebo BA	Mean DHT Testo BA	Treatment BA*	effect
1	E4 -	3.50	3.43	18.8		15.4
2	E4 -	4.19	3.16	12.4		9.28
3	E4 -	1.76	1.31	7.83		6.52
4	E4 -	1.20	1.26	4.71		3.45
5	E4 -	2.04	1.42	3.31		1.88
6	E4 -	1.07	1.82	7.06		5.23
7	E4 -	3.27	1.38	7.39		6.01
8	E4 -	2.95	3.13	4.90		1.76
9	E4 -	1.66	1.87	6.47		4.59
10	E4 -	2.79	2.57	13.1		10.5
11	E4 -	1.57	1.61	8.55		6.93
12	E4 -	1.96	1.52	13.1		11.6
13	E4 -	1.80	1.11	5.61		4.50
14	E4 -	2.17	2.01	10.1		8.10
15	E4 -	1.52	1.65	13.5		11.8
16	E4 -	1.64	1.16	5.92		4.76
17	E4 -	1.21	1.12	8.51		7.38
18	E4 -	2.32	0.98	10.6		9.65
19	E4 +	2.57	2.17	14.1		11.9
20	E4 +	0.51	3.35	6.95		3.60
21	E4 +	1.92	1.92	3.16		1.23
22	E4 +	2.04	1.79	4.94		3.15

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

All participants had some increase in DHT levels, although the increase did vary widely.

Table 5. Individual Estradiol levels Treatment A→B

Patient	APOE	E Baseline AB	Mean E Testo AB	Mean E Placebo AB	Treatment effect AB*
1	E4 -	60	101.7	70	31.7
2	E4 -	60	103.3	86.7	16.7
3	E4 -	80	93.3	80	13.3
4	E4 -	80	56.7	73.3	-16.7
5	E4 -	50	43.3	46.7	-3.33
6	E4 -	60	53.3	70	-16.7
7	E4 -	100	86.7	96.7	-10
8	E4 -	100	75	83.3	-8.33
9	E4 -	80	66.7	96.7	-30
10	E4 -	90	93.3	86.7	6.67
11	E4 -	80	93.3	56.7	36.7
12	E4 -	140	145	133.3	11.7
13	E4 -	90	91.7	130	-38.3
14	E4 -	50	45	60	-15
15	E4 +	90	90	73.3	16.7
16	E4 +	60	50	70	-20
17	E4 +	50	120	86.7	33.3
18	E4 +	120	118.3	93.3	25
19	E4 +	90	83.3	110	-26.7
20	E4 +	90	76.7	56.7	20
21	E4 +	80	80	90	-10
22	E4 +	80	121.7	83.3	38.3

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

11 participants had lower estradiol levels compared to placebo (yellow boxes), 2 participants started with high estradiol levels at baseline.

Normal reference range = 10-82 pg/mL

Table 6. Individual Estradiol levels Treatment B→A

Patient	APOE	E BA	Baseline	Mean E BA	Placebo	Mean E Testo BA	Treatment effect BA*
1	E4 -		70		80	210	130
2	E4 -		130		88.3	106.7	18.3
3	E4 -		100		88.3	106.7	18.3
4	E4 -		40		41.6	40	-1.67
5	E4 -		50		46.7	53.3	6.67
6	E4 -		40		45	46.7	1.67
7	E4 -		50		53.3	46.7	-6.67
8	E4 -		80		80	60	-20
9	E4 -		100		83.3	83.3	0
10	E4 -		50		60	100	40
11	E4 -		100		105	96.7	-8.33
12	E4 -		70		66.7	130	63.3
13	E4 -		90		66.7	70	3.33
14	E4 -		90		51.6	63.3	11.7
15	E4 -		90		68.3	143.3	75
16	E4 -		60		65	66.7	1.67
17	E4 -		130		173	210	36.6
18	E4 -		40		43.3	60	16.6
19	E4 +		80		80	83.33	3.33
20	E4 +		70		58.3	83.3	25
21	E4 +		110		91.6	73.3	-18.3
22	E4 +		120		93.3	93.3	0

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

5 participants had lower estradiol levels compared to placebo (yellow boxes), 3 participants started with high estradiol levels at baseline.

Normal reference range = 10-82 pg/mL

Table 7. Individual Body Mass Index (BMI) Treatment A→B

Patient ID	APO E	BMI Baseline AB	BMI The End of Testo AB	BMI The End of Placebo AB	Treatment effect AB
1	E4 -	27	23.1	25.2	-2.1
2	E4 -	21.2	21	20.9	0.1
3	E4 -	23.9	24.9	24.9	0
4	E4 -	23.1	23.3	23.3	0
5	E4 -	29.8	29.1	28.4	0.7
6	E4 -	30	28.5	29.7	-1.2
7	E4 -	25.6	25.4	25.2	0.2
8	E4 -	25.7	27	27.4	-0.4
9	E4 -	23.7	24.1	23.8	0.3
10	E4 -	28.2	29.5	29.4	0.1
11	E4 -	27.1	27.2	27.5	-0.3
12	E4 -	23	24.2	23.7	0.5
13	E4 -	25.8	25.1	25.6	-0.5
14	E4 -	24.6	24.4	24	0.4
15	E4 +	21.8	23.9	24.2	-0.3
16	E4 +	24.4	24.1	23.8	0.3
17	E4 +	28.7	27.1	27.2	-0.1
18	E4 +	26.6	26.2	25.7	0.5
19	E4 +	21.8	21.9	21.7	0.2
20	E4 +	32.4	32.5	32.3	0.2
21	E4 +	33.9	34.1	33.2	0.9
22	E4 +	32.6	32.4	32.5	-0.1

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

8 participants had lower BMI compared to placebo (yellow boxes), 8 participants were obese, 11 participants were overweight and 3 participants were underweight at baseline.

Normal reference range = 16-19 kg/m², cut off score of 22 kg/m² considered to be overweight, 27 kg/m² as obese (WHO).

Table 8. Individual Body Mass Index (BMI) Treatment B→A

Patient ID	AP OE	BMI Baseline BA	BMI The End of Placebo BA	BMI The End of Testo BA	Treatment effect BA
1	E4 -	25.3	26	26.4	0.4
2	E4 -	22.4	21.5	20.9	-0.6
3	E4 -	28.2	28.7	27.7	-1
4	E4 -	24.5	24.8	23.8	-1
5	E4 -	22.4	22.6	23.6	1
6	E4 -	27.8	28.5	26.5	-2
7	E4 -	21.1	20.1	20	-0.1
8	E4 -	22.4	22.3	21.8	-0.5
9	E4 -	25.5	25.2	25.7	0.5
10	E4 -	13.6	14.8	14	-0.8
11	E4 -	26.9	27.4	26.7	-0.7
12	E4 -	26.4	26.1	26	-0.1
13	E4 -	21.6	20.3	19.6	-0.7
14	E4 -	24.2	25.6	24.6	-1
15	E4 -	19.3	18.8	19.4	0.6
16	E4 -	26.6	25.9	25.2	-0.7
17	E4 -	31	31	31.4	0.4
18	E4 -	26.1	26.4	26.2	-0.2
19	E4 +	23.7	23.9	24.2	0.3
20	E4 +	30.1	30.3	29.4	-0.9
21	E4 +	20.9	23.3	24	0.7
22	E4 +	23.5	24.2	24.6	0.4

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

14 participants had lower BMI compared to placebo (yellow boxes), 4 participants were obese, 13 participants were overweight, and 5 participants were underweight at baseline.

Normal reference range = 16-19 kg/m², cut off score of 22 kg/m² considered to be overweight, 27 kg/m² as obese (WHO)

Table 9. Individual Body Fat Percentage Treatment A→B

Patient ID	AP OE	BF Baseline AB	BF The End of Testosterone AB	BF The End of Placebo AB	Treatment effect AB*
1	E4 -	25.4	24.7	25.4	-0.7
2	E4 -	17	15.8	17	-1.2
3	E4 -	19.3	20.7	21.5	-0.8
4	E4 -	18.1	18.5	20	-1.5
5	E4 -	33.1	32.5	31.7	0.8
6	E4 -	27.3	28.1	27.9	0.2
7	E4 -	21.5	20.6	21.8	-1.2
8	E4 -	20.2	23.6	22.1	1.5
9	E4 -	22.4	21.3	21.8	-0.5
10	E4 -	27	28.2	27.4	0.8
11	E4 -	26.2	26.8	29.1	-2.3
12	E4 -	19.9	21.2	20.1	1.1
13	E4 -	22.3	19.7	19.8	-0.1
14	E4 -	22.3	23.2	23.1	0.1
15	E4 +	14.5	18.7	19.4	-0.7
16	E4 +	24.6	23.9	23	0.9
17	E4 +	19.8	20.2	17.3	2.9
18	E4 +	23.1	23.5	24.3	-0.8
19	E4 +	22.2	21.5	21.1	0.4
20	E4 +	32.5	32	34.2	-2.2
21	E4 +	35.4	32.8	30.4	2.4
22	E4 +	32.5	29.5	28	1.5

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

11 participants had a lower body fat percentage compared to placebo (yellow boxes), 5 participants were obese at baseline.

Cut off score of 27% considered as obese (Gallagher et al. Am J Clin Nut 2000; 72:694-701)

Table 10. Individual Body Fat Percentage Treatment B→A

Patient ID	AP OE	BF Baseline BA	BF The End of Placebo BA	BF The End of Testo BA	Treatment effect BA *
1	E4 -	20.7	21.5	22.7	1.2
2	E4 -	19.5	19.1	17.5	-1.6
3	E4 -	27.3	27.9	27.8	-0.1
4	E4 -	22.3	23	21.7	-1.3
5	E4 -	15.9	16.6	19.2	2.6
6	E4 -	29.1	31.3	25.1	-6.2
7	E4 -	17.6	15.6	14.3	-1.3
8	E4 -	15.7	15.9	14.8	-1.1
9	E4 -	26.2	24.9	28.8	3.9
10	E4 -	2.5	5.7	3.6	-2.1
11	E4 -	25.4	28.8	26.6	-2.2
12	E4 -	20.5	22.2	19.8	-2.4
13	E4 -	15.8	15.4	13.5	-1.9
14	E4 -	21.9	23.2	23.4	0.2
15	E4 -	13.8	13	14.4	1.4
16	E4 -	28.8	27	27.5	0.5
17	E4 -	27.9	28.9	27.2	-1.7
18	E4 -	25.3	25.5	26.4	0.9
19	E4 +	22.9	22.9	25.6	2.7
20	E4 +	28.6	29.2	28.9	-0.3
21	E4 +	19.1	19.6	17.8	-1.8
22	E4 +	22.2	23.7	24.1	0.4

*Treatment effect = mean Testosterone treatment – mean Placebo treatment

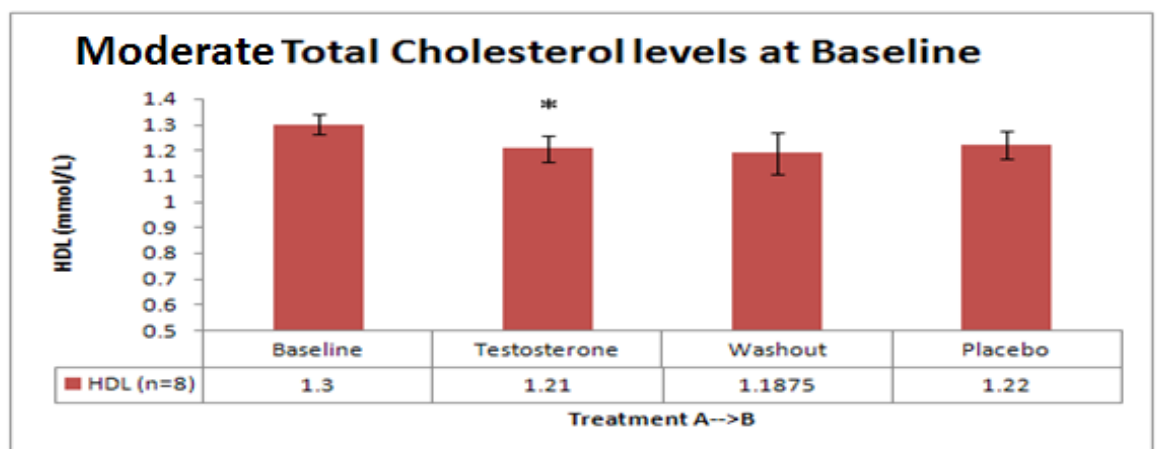
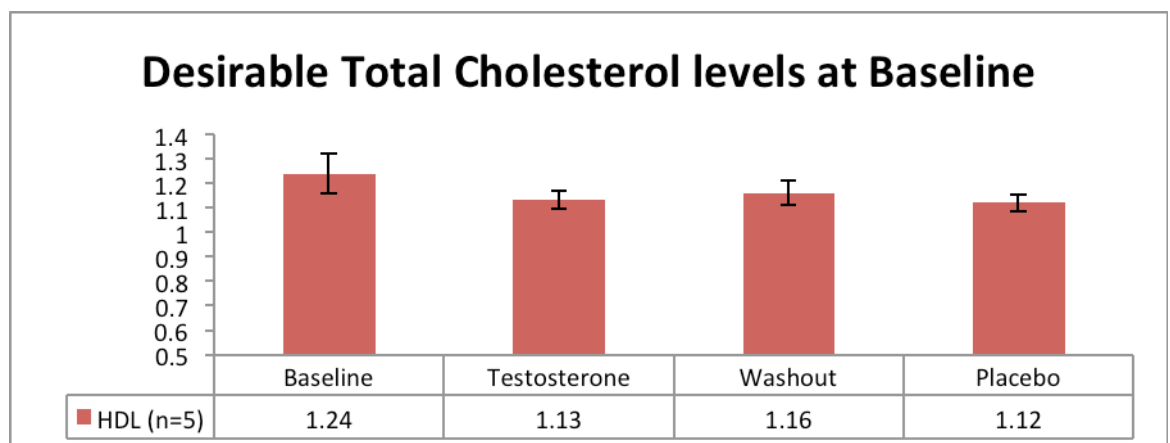
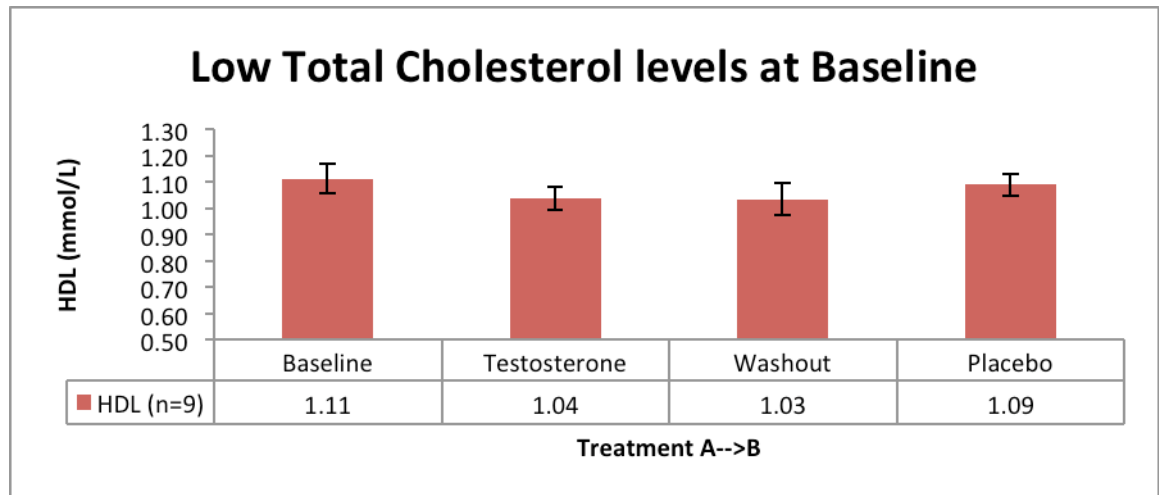
13 participants had a lower body fat percentage compared to placebo (yellow boxes), 5 participants were obese at baseline.

Cut off score of 27% considered as obese (Gallagher et al. Am J Clin Nut 2000; 72:694-701)

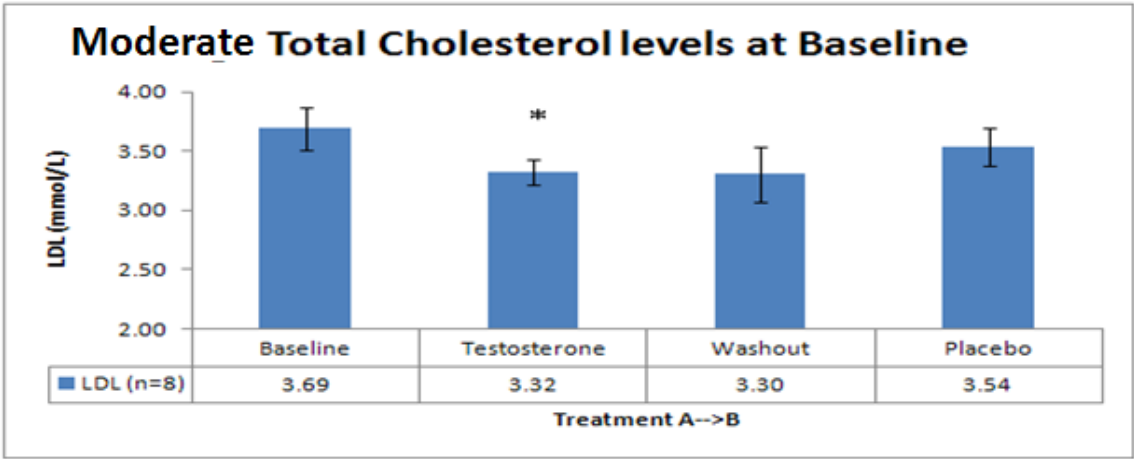
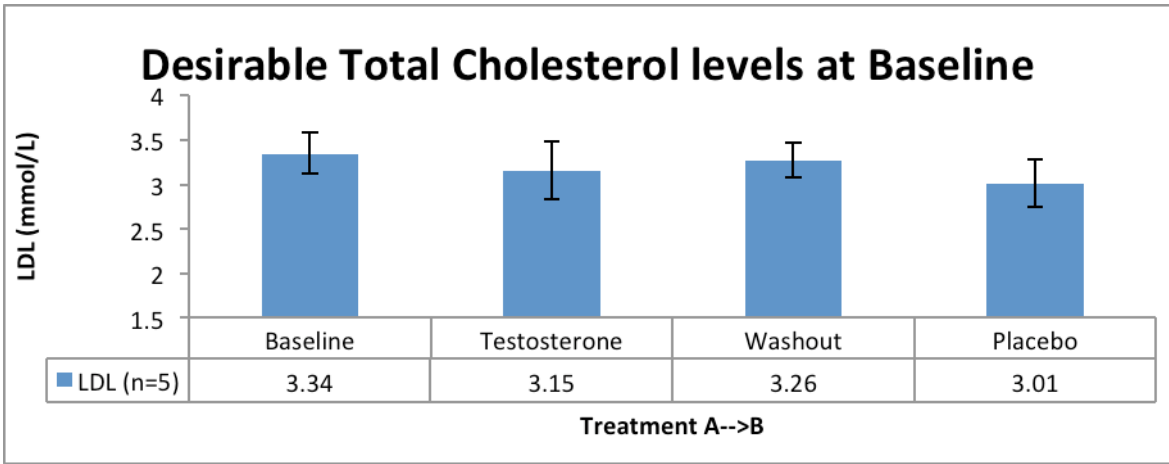
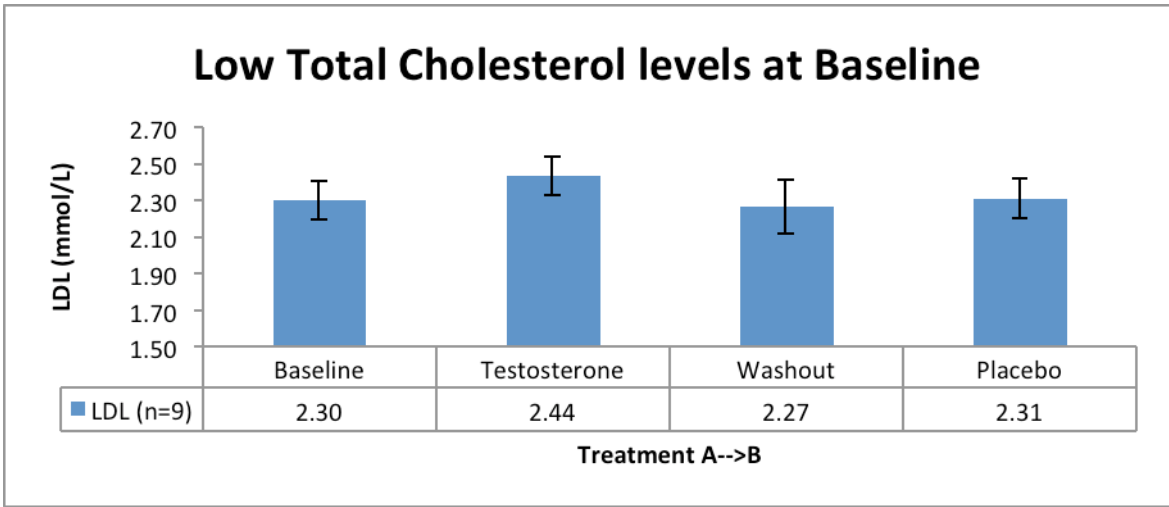
Lipid profile classification based on Total Cholesterol baseline threshold levels

Treatment A→B

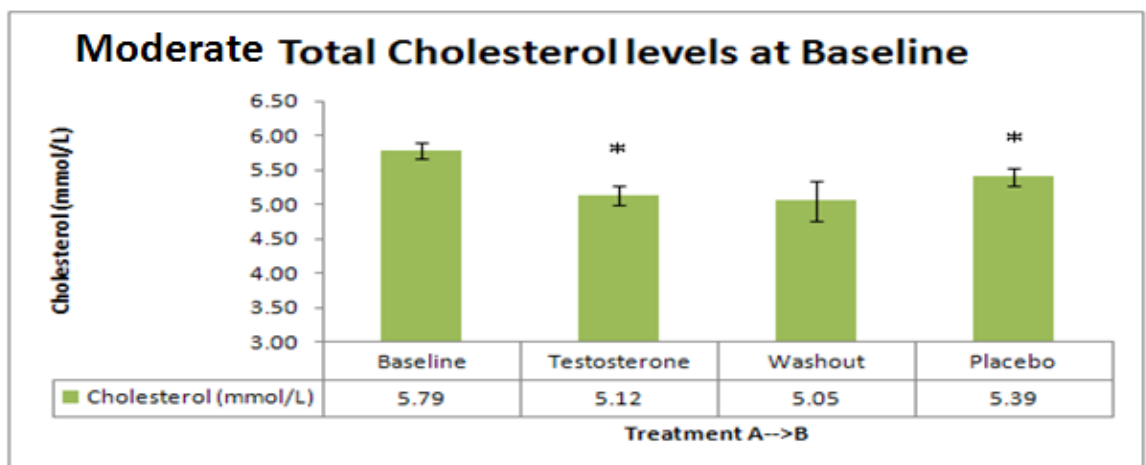
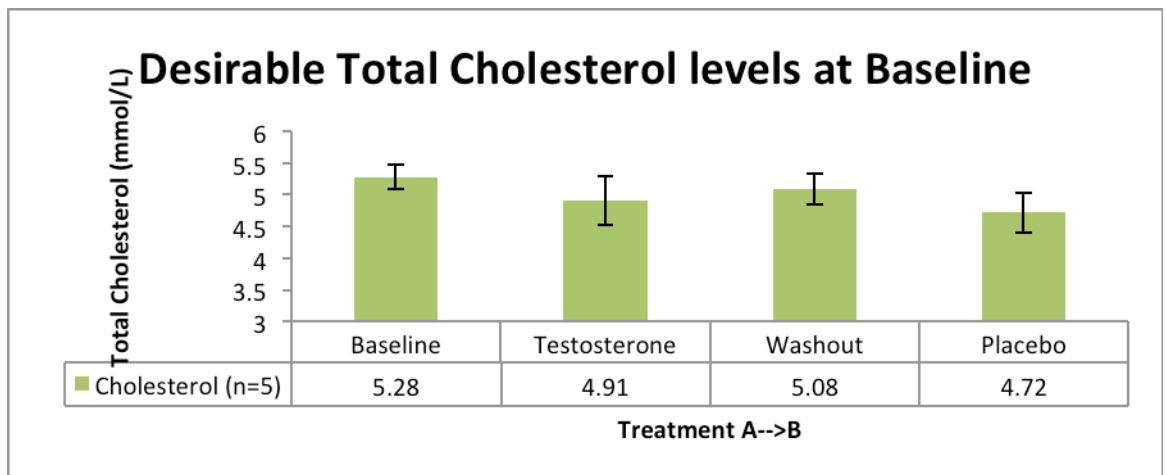
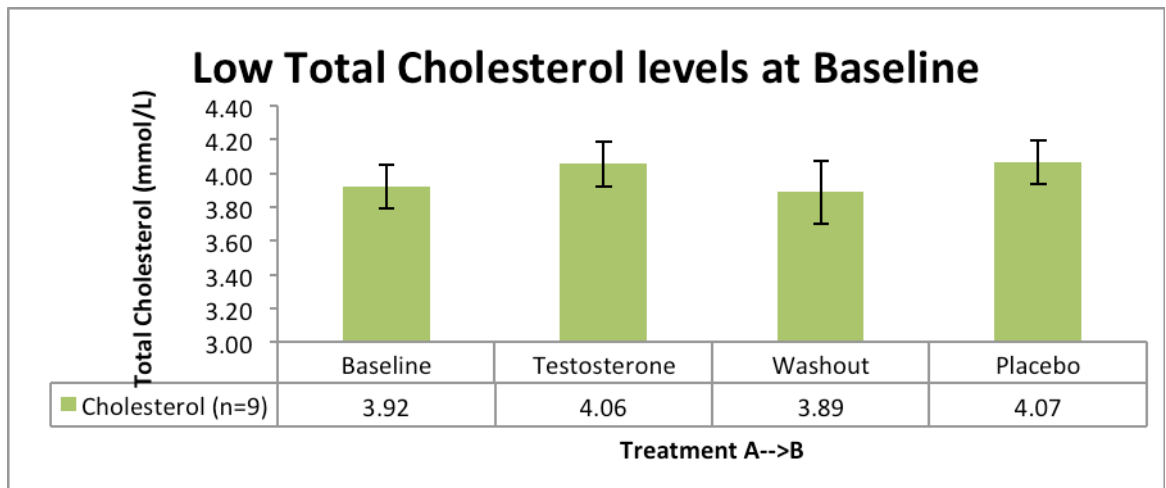
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LDL :

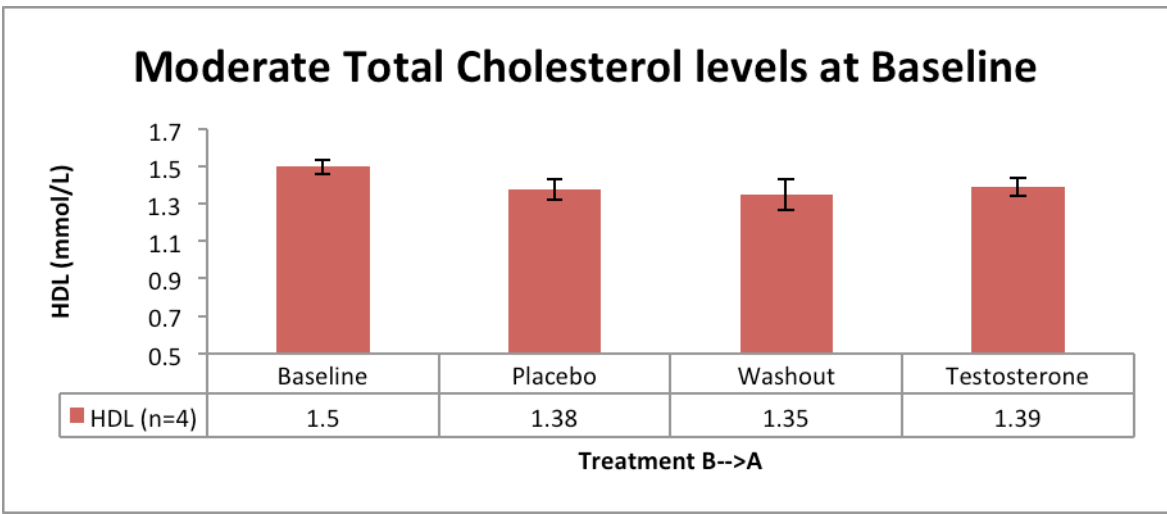
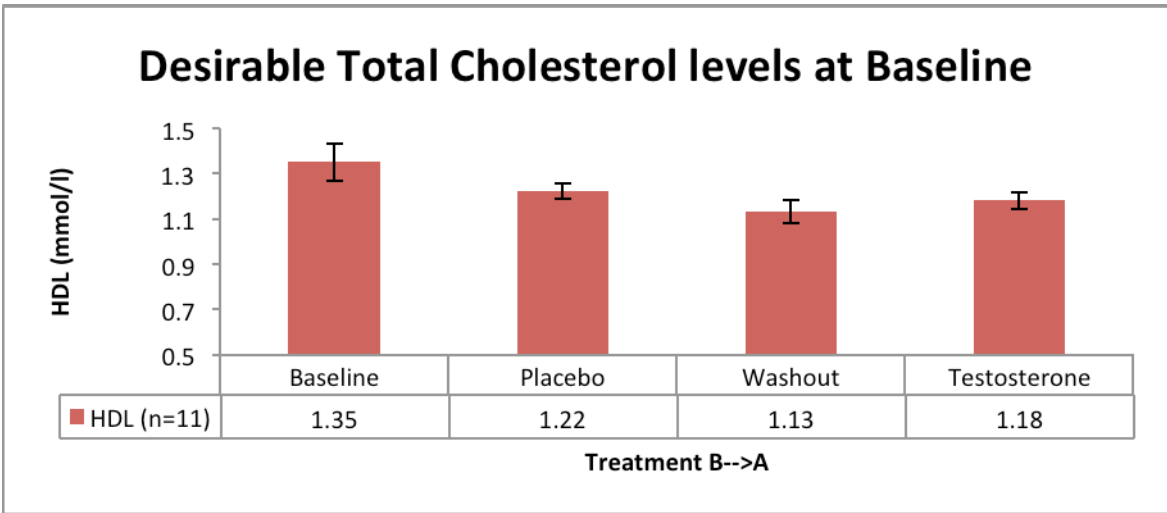
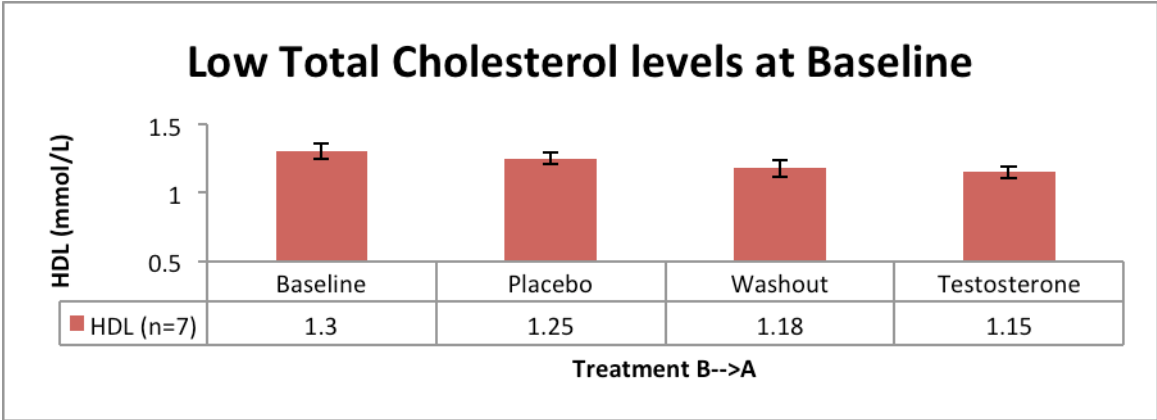


Total Cholesterol :

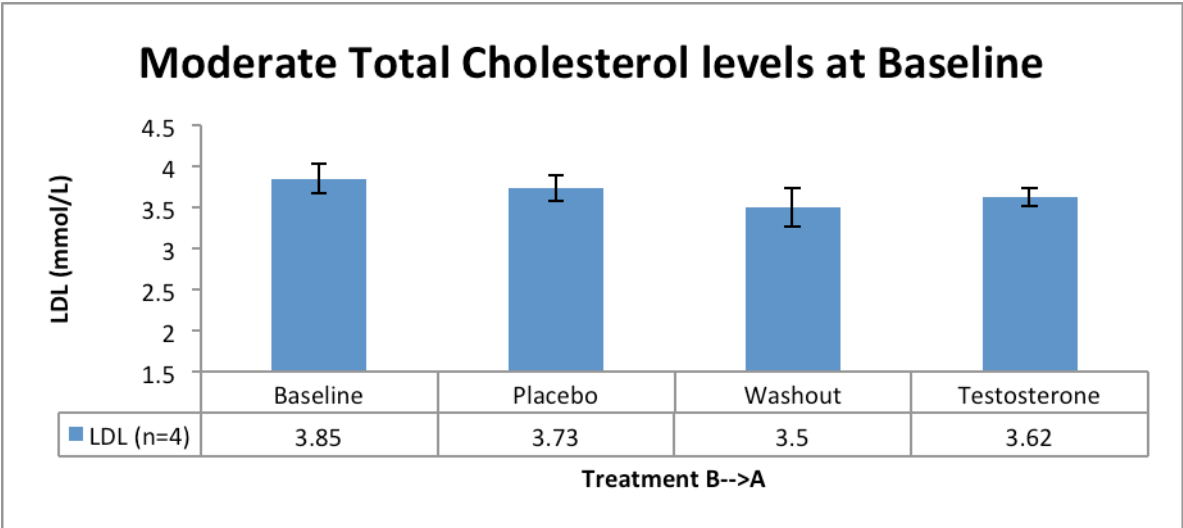
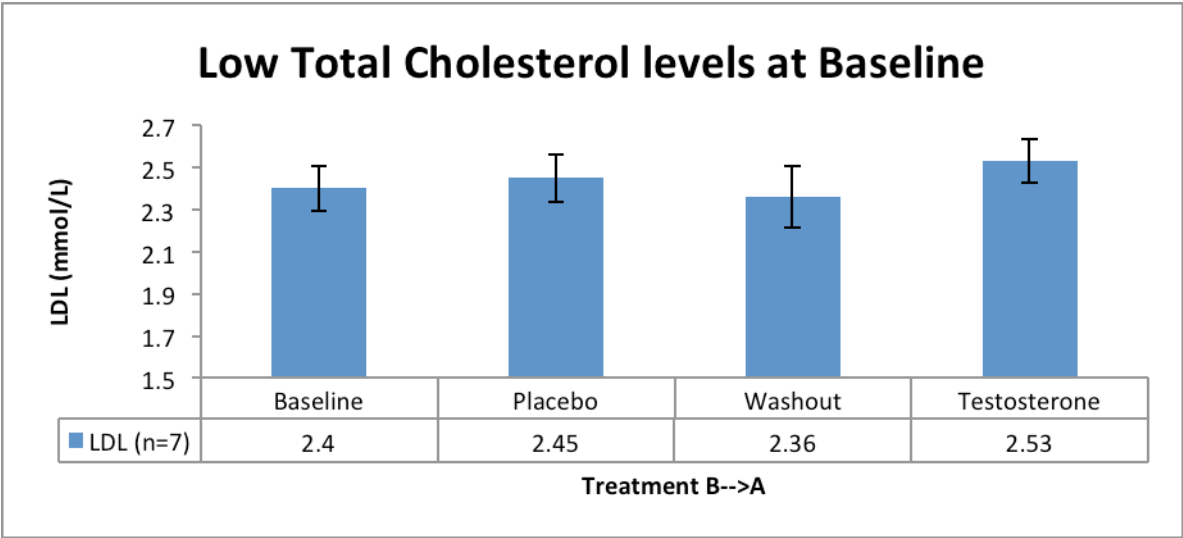


Lipid profile classification based on Total Cholesterol baseline threshold levels
Treatment B→A

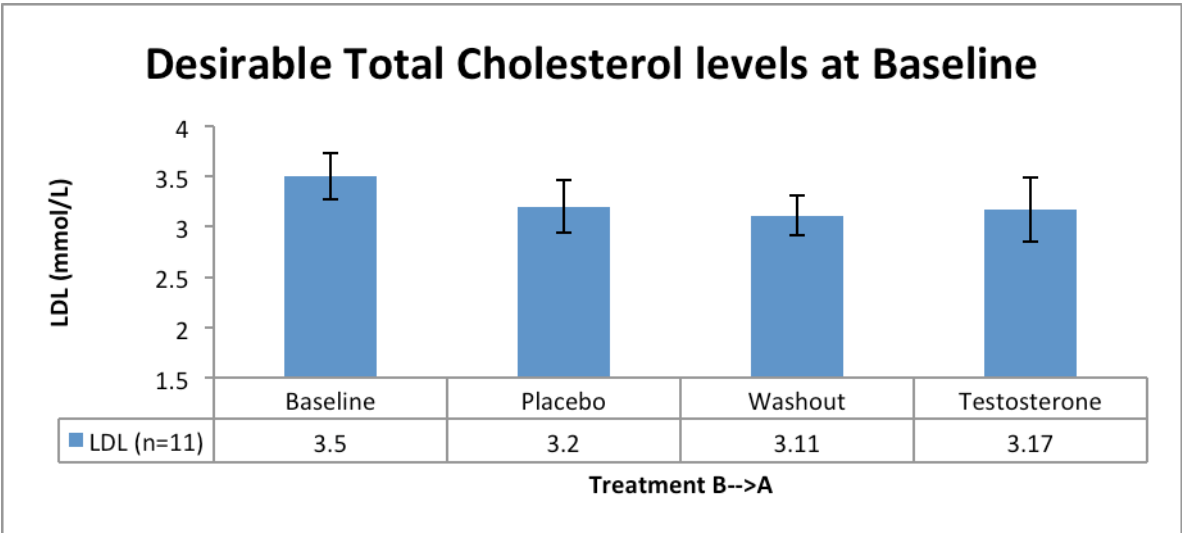
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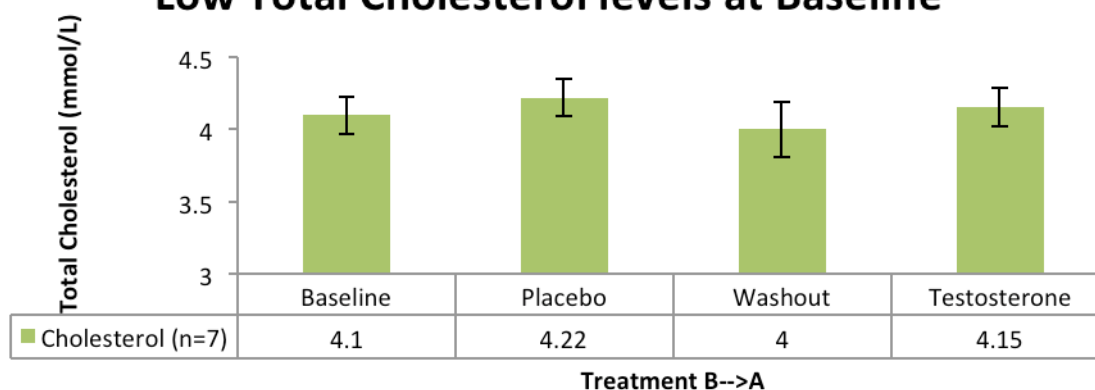
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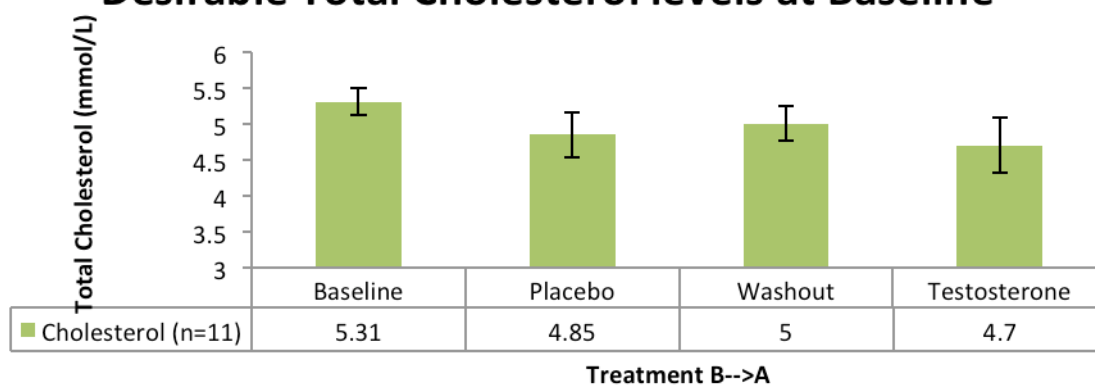
Total Cholesterol :



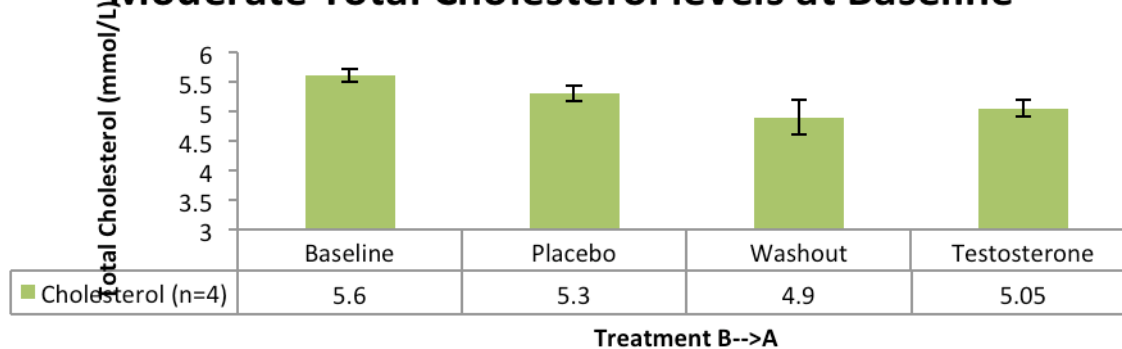
Low Total Cholesterol levels at Baseline



Desirable Total Cholesterol levels at Baseline



Moderate Total Cholesterol levels at Baseline



Total Cholesterol Treshold levels (at Baseline) :

< 4.6 mmol/L = low

$4.6 - 5.2$ mmol/L = desirable

$5.3 - 6.2$ mmol/L = moderate

≥ 6.2 mmol/L = high

All of the participants in the two arms of the study started with low, desirable, or moderate levels of total cholesterol levels at baseline.

From the graphs above, in treatment arm A→B, we can see that those who started off with higher than normal (desirable) levels of total cholesterol showed significant decreases in total cholesterol, LDL, and HDL following testosterone treatment. For those who started off with low and desirable total cholesterol levels at baseline, it may be that the treatment was not able to reduce levels further, because the body's metabolism compensated and produced cholesterol to maintain cholesterol levels above a certain minimum level. Therefore, these people were not likely to show any change. Thus, the results from the participants who started off with low and normal levels of total cholesterol may have masked any significant changes in participants who started off with moderate total cholesterol. Meanwhile, in treatment arm B→A, no significant changes were observed in those participants who started off with moderate total cholesterol levels. This may be due to the small number of participants, which was only 4. No significant changes were observed in those participants with low or moderate starting total cholesterol levels (as observed in the previous arm).